(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/011209 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/IL02/00628

(22) International Filing Date: 31 July 2002 (31.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/308,582 31 July 2001 (31.07.2001) US

(71) Applicant *(for all designated States except US)*: **YEDA RESEARCH AND DEVELOPMENT CO. LTD.** [IL/IL]; At The Weizmann Institute of Science, P.O. Box 95, 76 100 Rechovot (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHAUL, Yosef** [IL/IL]; Mercaz Shapira, 79 411 M.P. Sde Gat (IL). **GEIGER, Benjamin** [IL/IL]; 3 HaHitah Street, 76 352 Rechovot (IL). **PARAN, Nir** [IL/IL]; 16 Mendele Street, 75 209 Rishon LeZion (IL).

(74) Agent: **G. E. EHRLICH (1995) LTD.**; 28 Bezalel Street, 52 521 Ramat Gan (IL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/011209 A2

(54) Title: METHODS OF SENSITIZING HEPATOCYTE CELL CULTURES TO HEPATITIS INFECTION, BEADS COATED WITH HBV OR HCV POLYPEPTIDES AND METHODS OF USING SUCH BEADS TO MODEL VIRAL INFECTION OR DELIVER SUBSTANCES INTO HEPATOCYTES

(57) Abstract: There are provided methods of sensitizing hepatocyte cell cultures to hepatitis infection, beads coated with HBV or HCV derived ligands and methods of using same to model viral infection or deliver substances into hepatocytes.

METHODS OF SENSITIZING HEPATOCYTE CELL CULTURES TO HEPATITIS INFECTION, BEADS COATED WITH HBV OR HCV POLYPEPTIDES AND METHODS OF USING SUCH BEADS TO MODEL VIRAL INFECTION OR DELIVER SUBSTANCES INTO HEPATOCYTES

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to methods of sensitizing hepatocyte cell cultures to hepatitis infection. The present invention further relates to beads coated with HBV or HCV derived ligands and methods of using same to model viral infection or deliver substances into hepatocytes.

Five different viruses have been identified as causes of viral hepatitis. These include hepatitis A, B, C, D and E viruses. Of these, the viruses which cause the most serious infections are hepatitis B virus (HBV) and hepatitis C virus (HCV).

There are greater than 600,000 persistent carriers of hepatitis B virus (HBV) in the United States; the estimated total number of carriers in the world is greater than 200 million. HBV is implicated in both acute and chronic hepatitis. The disease is endemic in Asia and is increasing in prevalence in the U.S. and Europe. Chronic liver disease, resulting in significant morbidity and increased mortality, is a complication of infection in 1-10% of infected individuals. HBV infection is also correlated with the development of primary liver cancer.

HCV was recently shown to be the major causative agent of parenterally transmitted non-A, non-B hepatitis. It is estimated that 0.5-1% of the world population is infected with HCV, and in some developing countries the prevalence rate is up to 40%. Moreover, 40-60% of newly infected patients develop persistent HCV infections and are at risk of developing acute, fulminant hepatitis and various chronic liver diseases (including cirrhosis, chronic active hepatitis and in some cases hepatocellular carcinoma).

Although HBV and HCV have been identified and characterized, the development of new anti-viral strategies has been greatly hampered by the lack of convenient systems for infection and propagation in cultured hepatocytes.

Hepatitis Virus In Vitro

Hepadnaviruses, which include HBV, exhibit very narrow host and tissue specificity. Efficient infection by HBV is well documented for only humans and chimpanzees and, in cell culture, for primary hepatocytes from these hosts. Currently, biological assays for HBV have been limited to the experimental inoculation of chimpanzees, which are expensive and limited in numbers. Generic animal models exist, such as the woodchuck-woodchuck hepatitis B virus (WHV) and the duck-duck hepatitis B virus (DHBV) systems, but cannot support human HBV infection. Some rodent-based surrogate systems are available, although they are not infectable and require immunosuppression to prevent rejection of the human liver xenotransplants. Thus, little is known about the process of HBV attachment and infection of hepatocytes.

Investigation into HBV attachment and infection is hampered by the fact that the established human hepatocyte cell lines that retain the hepatocyte markers such as HepG2 and Huh-7 are refractive to HBV infection. Since these cell lines bind HBV via the pre-S1 domain, and efficiently transcribe the transfected HBV genome, attachment and entry are believed to be the restrictive steps. Human primary hepatocytes on the other hand are variably susceptible to infection and for only a short period after culturing (Mabit H et al J Hepatol 1996;24:403-412), but these systems are not practical because they are short-lived, require primary explanted liver from surgical biopsies, and are inconsistently susceptible.

In the absence of an efficient in vitro model of HBV infection, most research into HBV relies on primary or stable hepatocyte cultures transfected with viral genomes (Suri, D et al J Hepatol 2001;31:790-97; zu Pulitz J et al Virus Res 1997;52:177-82; Glebe D et al Intervirology 2001;44:370-78; Weiss

L et al Virology 1996;216:214-218; Shaotang R and Nassal M J of Virol 2001;75:1104-1116). Although these cultures can produce complete, infective virus particles, they are unsuitable for the study of infection.

A number of studies have reported attempts at productive HBV infection of hepatocyte cell lines, with generally unsatisfactory results: V8 protease-treated HBV virions were able to infect and replicate in HepG2 cells, but lost normal antigenic reactivity as a result of the proteolytic activity (Lu X et al, J Virol 1996;70:2277-85), rendering them unsuitable for studies of attachment and infection. In another study, Mabit et al found that HepG2 cells incubated with serum derived HBV particles expressed HBV antigens and transcribed viral RNA, but could not support viral replication or show other indicators of productive infection, and were very unstable in culture (Mabit, H et al J Gen Virol 1994;75:2681-89). Furthermore, these model systems were generally of low experimental reproducibility.

Early work with HBV in hepatocyte culture indicated that addition of Polyethylene glycol or DMSO to the culture medium improved the interaction between the cells and the virus. When human primary hepatocytes were treated with 2% DMSO prior to HBV infection, higher rates of infectivity (Gripon P et al J Virol 1988;62:4136-43 and Gripon P et al Virol 1993;192:534-40) and longer-term episomal replication of the virus (Rumin S et al J Viral Hepat 1996;3:227-38) were observed. DMSO treatment has also been used to improve DHBV infectivity and expression in primary duck hepatocytes (Galle PR et al Hepatology 1989;10:459-65), and it has been suggested that this solution mediator acts by maintaining the differentiated state of the cultured hepatocytes. Interestingly, in the duck hepatocyte-DHBV model, but not in human hepatocyte systems, the methylation inhibitor 5-Aza-2 Deoxycytidine, which has been used to induce lytic viral growth of Human Herpesvirus 8 in BCB cells (U.S. Pat No. 6,149,918 to McGrath et al), has also been used, with inconsistent results, to improve susceptibility to infection of primary duck hepatocyte cultures (Pugh JC and Simmons H, J Virol 1994;68:2487-94).

Hepatitis C virus, a positive sense RNA virus, also replicates poorly in vitro in human cells. Thus, there are no reliable in vitro models for the investigation of HCV infection, since no hepatocyte cultures permit robust in vitro infection with HCV. As with HBV, in-vitro propagation has been partially successful in primary hepatocyte culture (see, for example, Rumin S et al J Gen Virol 1999;80:3007-3018; Fournier C et al J Gen Virol 1998;79:2367-74), lymphoid cell lines and peripheral blood mononuclear cells. DMSO has also been used to improve HCV infectivity of human primary hepatocytes in culture (Rumin S et al J Gen Virol 1999;80:3007-3018) and is a constituent of a culture medium for primary mammalian hepatocytes recently disclosed in Intn'l Pat. Applic. No. EP 0972828A1 to Rumin S et al.

Thus, there remains a great need for a reliable and efficient stable human hepatocyte cell culture system capable of infection with both the hepatotrophic viruses HBV and HCV.

Hepatitis Virus Binding to Hepatocytes

One of the essential functions of virus surface proteins is the recognition of specific receptors on target cell membranes. The specific attachment of viruses to cells is the essential first step in virus entry into cells. The receptor specificity encoded in restricted regions of the virus surface structure can determine the virus host range, tissue tropism and pathogenesis. On the other hand, host cell receptors specific for different viruses have been identified as receptors for discrete physiologically important ligands. Therefore, viral binding to the host cell constitutes an aspect of virus-cell interactions critical to investigation, diagnostics and prevention of infection.

The HBV viral envelope is composed of a cellular originated membrane in which several virus coded surface proteins are anchored. These include the small HBsAgs (p24 and gp 28), the middle HBsAgs (gp33 and gp 36) and the large HBsAgs (p39 and gp 42). Of all the viral surface proteins, the large HBsAg of both avian and the mammalian hepadnaviruses, is believed to play a major role in mediating virus attachment and entry to hepatocytes: indeed,

antibodies against HBsAg are protective against HBV infection and the use of purified and recombinant HBsAg for diagnostic, prophylactic, vaccination and therapeutic purposes has been disclosed in U.S. Pat. Nos. 6,117,653; 6,110,706; 6,100,065; 6,072,049, all to Thoma; 6,099,840 to Thomas et al; 6,410, 009 to Galun et al.; 6,270,955 to Murray; and 6,297,048 to Jolly et al.)

Hepadnaviruses replicate almost exclusively in the liver. This liver tropism is believed to be determined, at least in part, by hepatocyte viral specific receptors and co-receptors. For example, the gp180 protein was recently identified as the putative duck hepatitis B virus (DHBV) receptor in duck hepatocytes. Suspected human HBV liver specific receptors include annexin V (Hertogs K et al Virology 1993;197:549-57), apolipoprotein H (Mehdi H et al J of Virol 1994;68:2415-24) and asialoglycoprotein (Treichel V et al, J Gen Virol 1994;75:3021-29).

Despite the identification of potentially important virus-host cell binding factors, the study and application of knowledge regarding HBV attachment and infection is hampered by the fact that the established human hepatocyte cell lines that retain the hepatocyte markers such as HepG2 and Huh-7 are refractive to HBV infection. Furthermore, no reliable methods are available to quantitatively evaluate virus attachment to the target cells. Thus, assessment of protective antibodies, and diagnostic methods have relied on detection of bound virus by PCR (Gerlich WH et al Vir Hepat Rev 1994;1:53-57) or immunofluorescence (Lu X et al J of Virol 1996;2277-85), labeled viral particles, anti HBsAg antibodies (Klingmuller, U. and Schaller, H J Virol 1993;67:7414-7422; Pontisso, P et al Virology 1989;173: 522-30.), and immunological assays with HBsAg or pre S2-coated beads (Neurath A.R et al Cell 1986;46: 429-36, and U.S. Pat. Nos. 5,620,844 and 5,204,996 to Neurath et al). However, none of the abovementioned methods demonstrate nor quantify HBV attachment at the single cell level.

Viral HCV envelope proteins critical to viral attachment and infection of hepatocytes have also been identified, among these the HCV E1, E2 and E1/E2

envelope proteins (Spaete RR et al, Virology 1992;188:819-30). Native and recombinant E1, E2 and E1/E2 proteins have also been proposed for use in vaccination, and in diagnostic and therapeutic applications (see U.S. Pat. Nos. 6,150,134 to Maertens et al; 6,297,048 to Jolly et al; and Rosa, D et al PNAS USA 1996;93:1759-63). However, since the HCV genome is RNA, the results of conventional PCR-based assays of binding must be interpreted with care (McGuiness P et al, Lancet 1994;343:551-52; Schroter, M et al J Clin Microb 2001;139:765-8). On the other hand, enzyme immunoassay and immunocytochemical tests currently in use have also been shown to be unreliable (Schroter M et al, J Med Virol 2001;64:320-24: Berger A et al J Clin Virol 2001;20:23-30) for screening.

Thus, it would be advantageous to have an accurate and reliable method of quantitating hepatitis virus-hepatocyte binding and interaction devoid of the abovementioned limitations. Such a method could not only provide real-time assessment of the critical initial stages of viral-host interactions and the possibility of quantitative, single cell attachment data, but could also be used for development of diagnostic and therapeutic tools directed at virus-host cell recognition and interaction.

Ligand-Mediated Targeting of Hepatocytes

The specificity of many interactions of the cell surface with elements present in the cellular environment, such as extracellular matrix, neighboring cells, macromolecules or microorganisms, provides the basis for many powerful investigative, diagnostic and therapeutic tools. Where a specific ligand has been identified, it can be used for detecting and isolating receptor-bearing cells and their receptors, defining and visualizing ligand-mediated processes, demonstrating anti-ligand immune activity, screening potential agonist/antagonist activities, and targeting of substances to specific cell populations.

Neurath et al, and others have disclosed the use of solid-phase immobilized HBsAg or anti-HBsAg antibodies, bound to agarose or

polystyrene beads, for immunoassay, detection of anti-HBV antibodies, purification of viral proteins and their antibodies (U.S. Pat. Nos. 5,798,206; 5,620,844; 5,565,548 to Neurath et al; Valinger Z Vaccine 1990;8:585-89). In other applications, cellular interactions with liver specific ligands, such as galactose and lactose (Cho CS et al, Biomaterials 2001;22:45-51, and van der Sluijs P et al Hepatology, 1986;6:723-28) and asialoglycoproteins (Watanabe Y et al J Biomat Sci Polym Ed 2000;11:833-48 and Martinez-Fong D et al, Hepatology 1994;20:1602-08) have been used to deliver bound substances to hepatocytes in vivo and in vitro.

Phagocytosis and endocytosis of microparticles coated with, or bearing specific ligands has also been demonstrated. Lymphocytes phagocytized CD8 immunobeads during magnetic sorting (Burkhardt O and Merker HJ Ann Anat 2002;184:55-60) and hepatocytes take up polybutylcyanoacrylate beads in vivo (Zhang ZR and He Q World Gastroent 1999;5:330-33). Defective hepatitis B viruses, or viral antigens having strong affinity and avidity for hepatocytes, have also been proposed as ligands for gene therapy and drug delivery (Wang L et al, Virus Res 2002;85:187-97; Wu J et al Front Biosci 2002;7:d717-25; and Protzer U et al PNAS USA 1999;96: 10818-823; recently reviewed by D Ganem, PNAS USA 1999;96:11696-97). Similarly, De Bruin WC et al used small HBsAg-bound 10 nm gold particles to investigate the role of endonexin II in virus attachment (de Bruin WC et al J Gen Virol 1995;76:1047-50). However, none of the abovementioned authors have demonstrated the direct visualization and quantitation of viral ligand-hepatocyte attachment and endocytosis of viral protein coated beads at the level of a single cell. Thus, there exists a need for a simplified, direct method of effecting and measuring binding between a hepatocyte and a ligand-coated substrate.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of obtaining cultured hepatocytes susceptible to viral infection, the

method effected by exposing the cultured hepatocytes to 5-Aza-2 Deoxycytidine and a solution mediator, to thereby obtain cultured hepatocytes susceptible to viral infection.

According to further features in preferred embodiments of the invention described below, the viral infection is an HBV or an HCV infection.

According to another aspect of the present invention there is provided a method of quantifying binding between a ligand and a hepatocyte, the method effected by contacting the hepatocyte with beads coated with the ligand and determining the amount of said beads bound to, or present in the hepatocyte to thereby quantify binding between the ligand and the hepatocyte.

According to yet another aspect of the present invention there is provided a method of delivering a substance into a hepatocyte, the method effected by contacting the hepatocyte with a bead coated with a ligand capable of binding the hepatocyte; and the substance, thereby introducing the substance into the hepatocyte.

According to still another aspect of the present invention there is provided a method of identifying a substance capable of modifying viral infection in hepatocytes, the method effected by contacting the hepatocytes with beads coated with a viral ligand capable of binding the hepatocytes, wherein said contacting is performed in a presence of the substance; and determining the amount of said beads bound to, or present in the hepatocytes to thereby determine the ability of the substance to modify viral infection of the hepatocytes.

According to still further features in the described preferred embodiments the solution mediator is DMSO.

According to yet further features in the described preferred embodiments the hepatocytes are HepG2 cells.

According to further features in the described preferred embodiments the hepatocytes are human hepatocytes.

According to yet further features in the described preferred embodiments the hepatocyte is a cultured hepatocyte.

According to still further features in the described preferred embodiments the hepatocyte is a cultured hepatocyte sensitized by contacting the hepatocyte with 5-Aza-2 Deoxycytidine and a solution mediator, to thereby obtain a sensitized hepatocyte.

According to yet further features in the described preferred embodiments the ligand is a viral protein.

According to further features in the described preferred embodiments the viral protein is HBsAg or a portion thereof.

According to still further features in the described preferred embodiments quantifying the beads is performed using a method of detection selected from the group consisting of microscopy, radioactivity, fluorescence, magnetic polarity, color detection and immuno-affinity.

According to yet further features in the described preferred embodiments contacting the hepatocyte is effected in a manner suitable for inducing endocytosis of the bead.

According to further features in the described preferred embodiments contacting the beads is performed in vitro or in vivo.

According to an additional aspect of the present invention there is provided a composition-of-matter comprising a bead coated with a hepatitis viral protein selected from the group consisting of HBV-HBsAg or a portion thereof and HCV E2 or a portion thereof; and a bioactive substance, wherein a size and/or shape of said bead is selected suitable for undergoing endocytosis.

According to yet an additional aspect of the present invention there is provided a pharmaceutical composition comprising the composition-of-matter described hereinabove and a pharmaceutically acceptable carrier.

According to further features in the described preferred embodiments the viral protein is a synthetic peptide.

According to further features in the described preferred embodiments the ligand is a synthetic peptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

According to yet further features in the described preferred embodiments the beads are selected from the group consisting of polystyrene beads, dextran beads, glass beads and metal beads.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel methods for sensitizing cultured hepatocytes to hepatitis viral infection, beads coated with HBV and HCV derived ligands, and an accurate and reliable method of directly quantitating hepatitis virus-hepatocyte binding and interaction, which can be used for development of diagnostic and therapeutic tools directed at virus-host cell recognition and interaction.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of

providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-1D depict the infection of 5-Aza-2 Deoxycytidine- and DMSO-treated HepG2 cells by HBV. Figure 1A is a series of confocal micrographs demonstrating the immunofluorescent detection of HBV antigens HBcAg (red, Rhodamine Red-X conjugated antibodies) and HBsAg (green, FITC-conjugated antibodies) in DMSO-treated (left panels) but not untreated (right panels) HepG2 cells, following incubation with HBV positive human serum. Yellow color (Merge) represents detection of colocalization of the viral HBcAg and HBsAg antigens in infected HepG2 cells. Figure 1B is a PAGE separation of PCR amplification products, demonstrating the efficient infection of DMSO-treated HepG2 cells by human HBV positive serum (lane 2). Virions contained only relaxed circular HBV DNA (RC-DNA) (lane 1), DMSO-treated cells contained both relaxed circular (RC-) and covalently closed circular (ccc-DNA) viral DNA (lane 2), while no viral DNA was detected in the untreated HepG2 cells (lane 3). Equal amounts of DNA were used for both treated and untreated controls (AML-2, lane 1 and 2). Figure 1C is a Southern blot analysis of total and extrachromosomal DNA demonstrating the presence of HBV replicative forms in DMSO-treated HepG2 cells incubated with human HBV positive serum. Lane 1 shows the migration of Mol. Wt. Markers. Lanes 2-11 show the presence of relaxed circular (RC), covalently closed circular (ccc) and single-strand (SS) viral DNA in total DNA [lanes 5-7, 5-14 days post infection (dpi), respectively] and extrachromosomal DNA (lane 11) from DMSO-treated HepG2 cells, indicating HBV infection. Note the absence of viral DNA forms from total (lanes 3 and 4) and extrachromosomal DNA (lane 10) of untreated,

and mock infected (lane M) cells, and from cells preincubated with 100 μM Lamivudine (3TC)(lane 8)14 hours after infection. Also note the blockage of infection in DMSO-treated cells by pre-incubation (4 hrs) of the HBV positive serum with MA 18/7 neutralizing monoclonal anti preS1 antibody (0.5 μg IgG) before infection (lane 9). Figure 1D is a series of confocal micrographs demonstrating the high-efficiency HBV infection of HepG2 cells pretreated with 5-Aza-2 Deoxycytidine and DMSO. Note the immunofluorescent detection of HBV antigens HBcAg (Core, green, FITC-conjugated antibodies) and HBsAg (red, Rhodamine Red-X conjugated antibodies) in 5-Aza-DC and DMSO-treated (TC-HBV and Serum HBV column) but not untreated (Control, center column) HepG2 cells, following incubation with HBV positive human serum (Serum HBV, right column) or pure concentrated virions (TC-HBV). The cellular protein Cadherin was detected equally well in all cells (Cad, blue, Cy5 conjugated antibodies). Note the infection and coexpression (yellow:Merge) of the viral HBcAg and HBsAg antigens in 100% the pretreated (TC HBV, Serum HBV) but none of the untreated (Control) cells.

FIGs. 2A-2C depict the expression, characterization and secretion of recombinant HBV sub-viral particles (SVPs) in stably transfected CHO cells. Figure 2A is a graphic representation of CsCl fractionation of sub-viral particles (SVPs) from CHO cells transfected with the HBV DNA (plasmid AL26). Purified particles were fractionated on CsCl gradient and the resulting fractions were assayed for HBsAg by radioimmunoassay using ^{125}I labeled anti HBsAg antibodies. HBsAg SVPs density was calculated to be 1.22 gr/ml, characteristic of the 22nm sphere HBsAg SVPs. The insert shows 22nm particles that were negative stained by Uranyl Acetate followed by Transmission Electron microscopy. Figure 2B shows a SDS PAGE and immunoblot analysis of the recombinant HBsAg, demonstrating the presence of all three HBsAg components. The composition of the various surface proteins was demonstrated by ^{35}SMet labeling on SDS PAGE (lane ^{35}S) and by western analysis with the specific antibodies for native preS1 (MA 18/7)(lane αS1),

preS2 (MA Q19/10)(lane αS2) and with polyclonal anti S antibodies (lane αS). Note the predominance of the small HBsAg proteins p24 and p28, in contrast with the larger, barely detectable p33, p36, p43 and p46 viral surface proteins. Figure 2C is a schematic representation of the viral HBsAg proteins S (p24), M (p33) and L (p43), with their corresponding antibody epitopes. The grey boxes represent the transmembrane region.

FIGs. 3A-3D are a micrographic demonstration of enhanced binding of SVP-beads on DMSO-treated HepG2 cells. Figures 3A and 3C are light micrographs (66X magnification) showing the binding of recombinant HBsAg component- conjugated 6 micron beads to untreated (Figure 3A) and DMSO-treated (Figure 3C) HepG2 cells. Control beads are conjugated with BSA (BSA). Note the overall increased affinity of recombinant SVP-conjugated beads for the HepG2 cells, as compared with BSA (Figure 3B) and the profound effect of DMSO pretreatment on viral-protein-specific attachment to HepG2 cells (Figure 3D), in both percentage of total cells binding beads (Total), and percentage of cells binding 4 or more beads (High);

FIGs. 4A-4H is a series of electron micrographs demonstrating DMSO-enhanced attachment to and endocytosis of recombinant SVP-conjugated beads by HepG2 cells. Figures 4A-4D are transmission electron micrographs showing the late stages (4A, 4B and 4D) and completion (4C) of engulfment of recombinant SVP-coated 6 micron beads by DMSO-treated HepG2 cells. Figure 4B is a higher magnification (1 micron bar equals 17 mm) of Figure 4A (1 micron bar equals 5 mm). Figures 4E-4H are scanning electron micrographs depicting efficient endocytosis of recombinant SVP-coated 6 micron beads by DMSO-treated HepG2 cells (4F), compared with the poor endocytosis by untreated (4E) cells. Various stages of beads internalization are demonstrated (4G and 4H), indicating tight association of the beads with the cell surface;

FIGs. 5A and 5B are a graphic representation demonstrating DMSO-enhanced binding of the recombinant SVP protein, preS1 region and 21-47 epitope of the HBsAg by HepG2 cells. 6 micron beads conjugated with one of

the following; BSA, SVPs, recombinant preS1 or synthetic peptide encompassing the amino acids 21-47 of preS1 were incubated with DMSO-treated (Figure 5A) or untreated (Figure 5B) HepG2 cells. Numbers represent the percentage of cells, out of the total population, binding more than 4 beads per cell. Bars indicate standard deviation. Note the greater than 50 fold increase in binding to DMSO-treated cells;

FIGs. 6A-6D depict the fine mapping of the preS1 attachment sequence to the QLDPAF motif. Figure 6A is an amino acid sequence comparison showing the wild type (WT) QLDPAF sequence, and a recombinant mutant, scrambled (mutant pre S1) sequence. Figure 6B is a immunoblot showing the detection of His-tagged wild type recombinant pre S1(WT) but not the scrambled recombinant mutant pre S1 (mut) protein by anti preS1 (MA 18/7) antibody (upper panel). Anti-6His antibody detected both wild type and scrambled mutant proteins equally well (bottom panel). Figure 6C is a graphic representation of the effect of scrambled QLDPAF motif on preS1 protein binding to HepG2 cells. 6 micron beads coated with wild type (WT) or mutant scrambled (mut) recombinant preS1 protein were incubated with DMSO-treated HepG2 cells, and scored for binding micrographically, as in Figure 3 (% of cell with greater than 4 beads per cell). Note the significant loss of attachment ability of mutant protein-conjugated beads. Preincubation of the cells with BSA (BSA) or mutant scrambled preS1 protein (mut) resulted in no significant reduction of wild type binding (competition). Figure 6D is an amino acid sequence comparison demonstrating the widespread distribution of the QLDFAP motif throughout diverse species. Database search revealed the presence of QLDFAP-like sequences in another HBV protein (HBV pX), and in many viral and bacterial (panel A) and eukaryotic (panel B) adhesion, attachment and fusion molecules;

FIGs. 7A and 7B demonstrate the binding by DMSO and 5-Aza-2-D-treated HepG2 cells of small sub-viral particles (sSVP) containing a non-preS1 and non-preS2 attachment region. Recombinant sSVPs were produced by

transfection of HEK 293 cells with the pMH8 plasmid. Figure 7A is Western blot with polyclonal anti HBsAg (αS) and monoclonal anti preS1 (MA 18/7) antibodies comparing the composition of the various HBsAgs in sSVPs and recombinant SVPs. The various HBsAgs (L, M and S) are indicated. Note the absence of MA 18/7 epitopes in the sSVP protein (right panel). Figure 7B is a graphic representation of the binding of sSVP and SVP- coated beads to HepG2 cells. 6 micron beads coated with SVP (SVP) or sSVP (sSVP) recombinant protein were incubated with DMSO (+) or untreated (-) HepG2 cells, and scored for binding micrographically, as in Figure 3 (% of cells with greater than 4 beads per cell). Note the retention of significant attachment ability of sSVP protein-conjugated beads, despite their lacking the preS1 and preS2 attachment regions. Also note that no significant binding was recorded with untreated (-) cells;

FIG. 8 is a graphic demonstration of inhibition of binding to Hep G2 cells, using pre S1, SVP and sSVP- coated beads. Figure 8A shows the effects of pre-binding incubation of the HepG2 cells with viral pre S1 protein: complete inhibition of binding of pre S1-coated beads (left panel, pre S1), and partial inhibition of binding of SVP-coated beads (middle panel, pre S1) and sSVP- coated (right panel, pre S1). Controls are pre-incubated with BSA (BSA), and results expressed as percentage of cells binding greater than 4 beads per cell. Note the low, but persistent (SVP- and sSVP-conjugated beads) portion of the binding which is unaffected by preincubation with even high concentrations of pre S1 protein (Figure 8A, bottom panel). Figure 8B shows the effects of incubation with anti pre S1 antibody MA 18/7 on Hep G2 binding of pre S1- and SVP- conjugated beads. Note the complete inhibition of binding of pre S1-coated beads (left panel) by the antibody (left panel, MA 18/7), and the low but significant portion of binding of SVP-coated beads (right panel) unaffected by the antibody (right panel, MA 18/7);

FIG. 9 is a micrographic demonstration of enhanced binding of HCV E2-beads on DMSO-treated HepG2 cells. Figures 9A and 9B are light

micrographs (66X magnification) showing the efficient binding of baculovirus-prepared HCV E2-conjugated 6 micron beads to DMSO-treated (Figure 9B) but not untreated (Figure 9A) HepG2 cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of novel methods of enhancing hepatitis viral infection of cultured hepatocytes. Specifically, the present invention can be used to obtain cultured human hepatocytes susceptible to HBV and HCV infection in vitro, for diagnostic and research applications. In addition, the present invention is also of bead compositions which can be used for identifying and screening substances modifying viral infection of hepatocytes, quantifying ligand binding to hepatocytes and for targeted delivery of bioactive substances to hepatocytes.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions. Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The study of hepatitis B and C virus attachment and infection is hampered by the fact that established human hepatocyte cell lines that express hepatocyte markers such as Hep G2 and Huh7 are refractive to infection. Since stable transformation of hepatocyte cell lines with the viral genome results in productive infection, hepatitis-transfected hepatocyte lines such as HepG2.21.5 (Gelbe D et al Intervirology 2001;44:370-78; Suri D et al J Hepat 2001;31:790-97; Weiss L et al Virology 1996;216:214-18) have become the standard for study of viral gene expression and anti-viral therapeutics. However, transfected

cell lines cannot provide a model for the study of early events in viral infection such as mechanisms of viral attachment and introduction into the hepatocyte, and generally have a very low production rate.

Human primary hepatocytes may be susceptible to hepatitis infection, but for only a short period after culturing (Mabit H et al J Hepatol 1996;24:403-412; Rumin S et al, J Gen Virol 1999;80:3007-18). However, these systems are not practical because they are short-lived, require primary explanted liver from surgical biopsies, and are inconsistently susceptible.

Exposure of primary hepatocytes to DMSO can enhance the susceptibility of these cultures to infection with HBV (Gripon P et al Virol 1993;192:534-40; Rumin S et al J Viral Hepat 1996;3:227-38) and HCV (Rumin S et al J Gen Virol 1999;80:3007-3018; Intnl Pat. Applic. No. EP 0972828A1 to Rumin S et al). While reducing the present invention to practice, it was uncovered that extended DMSO treatment of human cultured Hep G2 hepatocytes resulted in greatly enhanced susceptibility of the cultured hepatocytes to infection by serum-derived HBV, as demonstrated by the expression of viral antigens and replicative form viral DNA (Figures 1A-1C). As is further described herein, it was observed, for the first time, that pretreatment of the Hep G2 cells with DMSO and the methylase inhibitor 5-Aza-2 Deoxycytidine resulted in even greater susceptibility to HBV infection, with 100% active infection of the sensitized hepatocytes (Figure 1D). Thus, the method of the present invention can be used to develop hepatocyte culture systems for viral infection with, for example HBV and HCV, enabling investigation of primary hepatocyte-virus interaction and the screening of drugs, such as anti-viral prophylactic agents. By providing a method for producing cultured hepatocytes susceptible to viral infection the present invention overcomes the limitations of restricted longevity, availability and variability inherent in primary hepatocyte culture, and the poor applicability of transfected hepatocyte cultures to investigation of hepatocyte-virus interactions.

Thus, according to one aspect of the present invention, there is provided a method of obtaining cultured hepatocytes susceptible to viral infection. The method is effected by exposing the cultured hepatocytes to 5-Aza-2 Deoxycytidine and a solution mediator.

As used herein, the term "solution mediator" refers to a substance which improves the interaction of molecules such as proteins in a solution, with a solvent, resulting in enhanced solubility of the molecules. Examples of solution mediators are dimethylsulfoxide (DMSO), methyl sulfonyl methane (MSM) and polyethyleneglycol (PEG).

As used herein, the term "cultured hepatocytes" refers to hepatic or hepatic-derived cells which can be maintained in vitro under appropriate conditions (e.g. suitable medium, temperature, CO_2 saturation) preferably in a proliferative state. It will be appreciated that "cultured hepatocytes" are distinct from "primary hepatocyte cultures", which are derived from tissue removed from live hosts, and cannot be induced to proliferate. Examples of such cultured hepatocytes are, for example, established human hepatoma cell lines such as Hep G2 and Huh7.

As used herein, the phrase "susceptible to viral infection" when used in context with a cultured hepatocyte, refers to the ability of the hepatocyte to display signs of viral replication and/or expression of viral genes following exposure to virus particles, attachment, and internalization of virus particles.

While reducing the present invention to practice, it was observed that Hep G2 cells pretreated with 2% (V/V) DMSO and 100 mM 5-Aza-2 Deoxycytidine for 10-14 days are susceptible to infection in vitro by both serum-derived HBV viruses and pure, culture-derived HBV viruses (Example 1). Whereas previous attempts to infect cultured human hepatocytes with HBV have resulted in inconsistent infection (see, for example, Mabit H et al J Gen Virol 1994;75:2681-9), all of the DMSO and AzaDC-treated cells, and none of the controls, expressed both the core (HBcAg) and S (HBsAg) viral proteins (Figure 1D). Furthermore, the methods of the present invention achieved

infection of the hepatocytes with native and purified virus particles (Figure 1D), avoiding the severe protease treatment of virions required by Lu et al (Lu X et al J Virol 1996;70:2277-85).

According to one preferred embodiment of the present invention, the solution mediator is DMSO, which is utilized in final concentration of 0.1-5.0% (V/V), preferably 1.0-2.0%, most preferably 2.0%, along with the 5-Aza-2 Deoxycytidine which is utilized at a final concentration of 10-200 mM, preferably 100 mM. Methods of pretreatment of cultured hepatocytes with AzaDC and DMSO suitable for use with the present invention are described in more detail in the Examples section which follows.

As detailed hereinabove in the Background section, the lack of viable in vitro cell culture models of human HBV and HCV infection has impeded understanding of hepatocyte-virus interaction and, subsequently, the development of drugs effective in preventing infection. The method for obtaining cultured hepatocytes susceptible to HBV and HCV infection of the present invention can be used to establish viable cultures of viral infected human hepatocytes, devoid of the inconsistency and poor availability of primary human hepatocyte culture.

Thus, according to another preferred embodiment of the present invention, cultured hepatocytes are human hepatocytes. More preferably, the human cultured hepatocytes are Hep G2 cells. Preferably, the viral infection of the method of the present invention is a human hepatotropic virus infection such as HBV or HCV.

The cultured human hepatocytes susceptible to viral infection may also be used as a powerful screening tool for evaluating the anti-viral effectiveness of a compound, e.g., a drug or an antibody to be administered to a patient.

Such an in vitro assay can be effected by providing a sample of susceptible hepatocytes; contacting the sample with virus and the compound to be tested, (e.g., for at least 15 minutes); and measuring parameters of viral infection, such as viral protein expression and DNA replication in the sample.

Some compounds may require contacting the hepatocytes for longer than 15 minutes, e.g., 1 hour, 3 hours, 24 hours, or up to several days, in order to determine the effect of the compound on the viral infection of the hepatocytes. A decrease in, for example, viral attachment and/or viral replication in the hepatocytes in the presence of the test compound compared to that in the absence of the same compound indicates that the compound is likely to be effective in preventing infection in vivo. Such a screening assay is adaptable to automated high throughput technology employing large numbers and samples of cell cultures (for a review of high throughput screening techniques, see Kenny, BA et al Prog in Drug Res 1998;51).

Indeed, while reducing the present invention to practice, it was surprisingly observed that susceptible Hep G2 cells exposed to HBV in the presence of the HBV RTase inhibitor Lamivudine (Example 1, Figure 1C, lane 8), or an infection blocking anti-pre S1 antibody (Example 1, Figure C, lane 9) remained devoid of viral replicative form DNA, indicating inhibition of infection and sensitivity of the method to anti-viral activity. Both Lamivudine (Colledge D et al Hepatology 1997;26:216-25) and the anti-preS1 antibody (Neurath et al Vaccine 1989;7:234-36) have proven anti-viral activity.

Alternatively, the infectiveness of various strains of virus in human hepatocytes may be assessed in a similar screening assay, wherein viral infection of susceptible cells is compared between strains having previously demonstrated, and those of undetermined, infectivity.

As described in detail hereinabove, the current methods of assessment of hepatocyte infection by HBV or HCV rely on detection of bound virus by PCR or immunofluorescence, labeled viral particles and immunological assays with HBsAg and the HCV E1, E2 and E1/E2 envelope proteins. However, due to the minimal amounts of viral DNA and RNA associated with cells in early stages of infection of cultured cells, and the inconsistency of the immunological and enzyme immunoassay techniques, no reliable methods are available to quantitatively evaluate viral attachment and early infection of hepatocytes.

Furthermore, none of the abovementioned methods demonstrate nor quantify viral attachment at the single cell level.

While reducing the present invention to practice, it was observed that beads conjugated with HBV and HCV viral proteins bind to cultured hepatocytes sensitized by the method of the present invention, but not to unsensitized hepatocytes (Example 2), enabling to quantitatively model virus attachment.

Thus, according to another aspect of the present invention there is provided a method of quantifying binding between a ligand and a hepatocyte. The method is effected by contacting the hepatocyte with beads coated with the ligand and determining the amount of the beads bound to, or present in the hepatocyte, thereby quantifying binding between the ligand and the hepatocyte.

Preferably, the hepatocyte culture utilized by this aspect of the present invention is presensitized to infection as described hereinabove.

As used herein, the term "binding" refers to a specific affinity-based interaction between two or more molecular entities, resulting in attraction and varying degrees of physical association. It will be appreciated that the binding may be of varying strengths, typically defined by the strength of solvent washings which the bonded entities can withstand.

As used herein, the term "ligand" refers to a molecule which is recognized by, and has affinity for, another molecule or molecular structure, typically termed a "receptor". Typically, the interaction between the ligand-receptor pair is of varying degrees of specificity, occurring at the surface of cells or membranes, and initiating specific biological processes such as signal transduction or internalization of ligands.

As used herein, the term "bead" refers to any one of microspherical solid substrates suitable for conjugation with a putative ligand, having shape and dimensions affording specific interaction and binding between the ligand and a ligand-recognition entity on the surface of a hepatocyte. Such beads are commercially available in a variety of shapes, sizes and materials, such as 6

micron microspherical polystyrene beads (Polysciences, Warrington PA, USA). The beads can be polystyrene beads, dextran beads, glass beads or metal beads (such as 10 nm colloidal gold), preferably, the beds utilized are polystyrene beads.

Methods of coating beads with ligand molecules, such as, for example proteins, are well known in the art. Briefly, the ligand is prepared in solution and adsorbed on a bead, such as, a polystyrene bead, which is pretreated or fabricated to include binding sites on its surface. The bead surface is thereafter contacted with a blocking solution (e.g., protein containing solution), for example, ethanolamine, gelatin BSA or powdered milk, in order to saturate the unbound binding sites of the bead. Thereafter, the substrate is washed with a buffered solution, the buffer is removed, and the beads are resuspended in storage buffer until use (preparation of beads, and binding of viral antigens is detailed in the Material and Methods section of the Examples section which follows).

The use of ligand-coated beads for modeling viral infection has several advantages: (i) beads are easily detected, and their attachment is not followed by immediate internalization into the cells, hence more easily quantifiable, thus, the viral ligands and their cellular receptors are more easily identified; (ii) binding to cell surface receptors is resolved at the level of a single cell; (iii) susceptible cells, binding viral ligand-coated beads, can be easily sorted out of a heterogeneous cell population, and categorized according to relative binding capacity; (iv) high concentrations of ligand on bead surfaces can enhance binding avidity and permit detection of otherwise weak binding interactions; and (v) beads, and thus ligand-hepatocyte interactions, are easily visualized in real time.

Coated beads have been used for analysis of adhesion mediated interactions (Levenberg, S et al J Cell Sci 1998;111:347-357), and for detection of immune response to HBV infection (Neurath A.R et al Cell 1986;46: 429-36, and U.S. Pat. Nos. 5,620,844 and 5,204,996 to Neurath et al).

Neurath et al disclosed solid substrates, such as polystyrene beads coated with hepatitis B antigens or anti-hepatitis B immunoglobulins, for detection and separation of anti-hepatitis B antibodies, or hepatitis B viral proteins from fluids, and for evaluation of anti-HBV vaccination (US Pat. No. 5,565,548 to Neurath et al). Similarly, secondary antibodies have been bound to solid substrates such as styrene beads for enzyme immunoassay of HIV, and for detection of response to viral infection by flow cytometry (Chersi A et al Hum Immunolog 2000;61:1298:306). However, the abovementioned prior art does not disclose or suggest binding of coated beads to cells.

The present inventors have demonstrated for the first time that beads coated with HBV sub-viral particle (SVP) proteins (Example 2), and beads coated with recombinant pre S1 protein, in particular, a synthetic polypeptide representing the antigenic 21-47 amino acid epitope of the HBV preS1 protein (SEQ ID NO: 1) specifically bind cultured, sensitized hepatocytes. The present inventors have also demonstrated that the coated beads described herein are internalized by the cells through endocytosis (Example 3, Figures 4G and 4H).

Thus, binding between a ligand-coated bead and a hepatocyte can be determined by a method capable of detecting beads bound to, or internalized within the hepatocyte. Detection can be effected on the basis of visual observation, for example, microscopically or by color detection of colored beads, by the behavior of magnetically polarized beads in a magnetic field, by detection of emission, such as radioactivity or fluorescence from the beads, or by interaction with immune reactive substances bound to the beads. Methods for detection are well known in the art, such as the confocal laser and fluorescent microscope (see Cho, CS et al Biomaterials 2001;22:45-51, Xu Z et al J of Virol 1997;71:5487-94, and Materials and Methods section hereinbelow), electron microscope (as detailed in Materials and Methods section hereinbelow) and magnetic bead separator (Dynal Biothech, Oslo Norway and Miltenyi Biotec, Bergisch-Gladbach, GmbH). In one preferred embodiment, quantifying the beads binding to the hepatocyte is performed by

direct microscopic observation and counting of the bound beads, according to the criteria: cells remaining unoccupied (0 beads per cell), cells attaching 1-4 beads, and cells attaching more than 4 beads each.

Detection and quantifying of binding between viral ligand-coated beads and hepatocytes can be used to screen for substances useful in preventing viral infection. Putative anti-viral compounds can be added to the culture medium before, during and/or after contacting with the viral-ligand coated beads, and binding and/or internalization of the beads compared to that of cells without added substances. Inhibition of beads binding to, or internalized into the hepatocytes would likely indicate an anti-infective effect of the substance. Thus, according to another aspect of the present invention, there is provided a method of identifying a substance capable of modifying viral infection in hepatocytes. The method is effected by contacting the hepatocytes with beads coated with a viral ligand capable of binding the hepatocytes, in the presence of the substance, and then determining the amount of the beads bound to, or present in the hepatocytes, as detailed herein, to determine the infection-modifying ability of the substance.

Preferably, the viral ligand is HBsAg, and more preferably, a synthetic polypeptide representing the antigenic 21-47 amino acid epitope of the HBV preS1 protein (SEQ ID NO: 1), containing the QLDPAF sequence (Example 3). Thus, in one preferred embodiment the ligand is a synthetic peptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

Ligand-cell surface interactions have been used for targeting delivery of drugs, and other bioactive substances to specific cell populations, such as lymphocytes. Hepatocyte -specific ligands such as asialoglycoprotein moieties (Martinez-Fong D et al Hepatology 1994;20:1602-8; van der Fluijs P et al Hepatol 1986;6:723-28), polybutylcyanoacrylate (Zhang ZR and He Q World J of Gastroentero 1999;5:330-33) and others (for review, see Wu J et al Front Biosci 2002;7:d717-25) have been associated in lipoproteins and microparticles with cell modulators such as colchicine, cytochlasin B, taxol and valaciclovir

for liver-specific targeting of these drugs. Similarly, gene therapy using recombinant, defective hepatitis B viruses, taking advantage of strict HBV hepatotropism, has been recently proposed (Wang L, et al Virus Res 2002;85:187-97; Protzer U et al PNAS USA 1999;96:10818-823). However, the asialoglycoprotein moieties tend to be unstable and of limited solubility. The use of ligand-coated beads for targeting drugs to hepatocytes was not disclosed.

Since it was observed, for the first time, that 6 micron polystyrene, SVP-coated beads bound to the surface of sensitized Hep G2 hepatocytes are efficiently internalized via endocytosis (Example 3, Figures 4G and 4H), the coated beads of the present invention can also be utilized as delivery vehicles.

The strict specificity of this endocytosis is demonstrated by the absence of internalization by non-sensitized hepatocytes, and by the failure of the sensitized cells to internalize Concanavalin A-coated beads, although attachment was detected.

Thus, according to another aspect of the present invention there is provided a method of delivering a substance, such as a drug, into a hepatocyte. The method is effected by contacting the hepatocyte with a bead coated with a ligand capable of binding the hepatocyte, and the substance, thereby introducing the substance into the hepatocyte.

It will be appreciated that coating of the bead with the substance to be delivered is performed essentially as described herein for the ligand. The bead can be exposed to the ligand prior to, simultaneously with, or after exposure to the ligand, prior to the blocking of available binding sites. Ligand concentration can be greater, equal or lesser than substance concentration on the bead. Effective ratios of substance to ligand will vary with different substances and ligands, and specific applications.

Substances bound to polymers of asialoglycoprotein, and neoglycoprotein have been observed targeting the liver in in vivo studies (Wu J et al Front Biosci 2002;7:d717-25; van der Sluijs P et al Hepatology

1986;6:723-28). Thus the method of delivering a substance to a hepatocyte of the present invention is well suited for delivering substances, such as anti-viral drugs, in vitro, to cultured hepatocytes, and in vivo, to liver cells in living organisms. The beads can be administered by intravenous injection or by release from a bolus introduced in or near the liver or liver blood supply.

Thus, in one preferred embodiment of the present invention, contacting the hepatocyte is performed in vivo. In another embodiment, contacting the hepatocyte is performed in vitro.

It will be appreciated that a wide variety of substances can be coated onto beads, and delivered to hepatocytes using the method of the present invention. Indeed, Wu et al (Front Biosci 2002;7:d717-25) and Ganem (PNAS USA 1999;21:11696-697) have proposed liver-specific targeting of DNA for genetic manipulation of hepatocytes. Protein coating of beads is widely known in the art, and the methods described herein can be used to coat beads with immunoglobulins, peptide and proteinaceous drugs. Nucleic acids can also be coated onto solid substrates using methods known in the art (see, for example, Pietrasanta L et al PNAS USA 1999;96:3757-62).

The substance to be delivered can be an antibody, a toxin, a nucleic acid or a drug.

As used herein, the term "antibody" includes intact immunoglobulin molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv that are capable of binding to antigens. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme

pepsin without subsequent reduction; F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art (see for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference). Examples of antibodies that can be used in the method of the present invention are anti-viral antibodies, which can be introduced into the hepatocytes to disrupt infectious processes.

As used herein, the term "toxins" refers to any of a group of poisonous substances typically active in disrupting one or more metabolic pathways of living cells. Examples of toxins commonly administered therapeutically are pertussis toxin (e.g., for gastroparesis) and botulinum toxin (bladder carcinoma). Furthermore, many toxins are used in cancer therapy, to weaken or kill cancerous cells. Beads coated with such toxins, and liver specific ligands, can be targeted to cancerous cells in treatment of hepatocarcinoma and other neoplasms of the liver.

As used herein, the term "nucleic acid" refers to oligo and/or polynucleotides of ribonucleic acid or deoxyribonucleic acid, or combinations thereof. Examples of nucleic acids that can be introduced into hepatocytes are, but are not limited to antisense or ribozyme RNA, DNA sequences encoding biologically active proteins such as receptors, enzymes and cofactors, and oligonucleotides recognizing regulatory sequences. The method of the present invention can be used for hepatocyte targeting in in vivo gene therapy. In in vivo gene therapy, target cells are not removed from the subject rather the

genetic material to be transferred is introduced into the cells of the recipient organism in situ, that is, within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired in situ [Culver, 1998. (Abstract) Antisense DNA & RNA based therapeutics, February 1998, Coronado, CA].

As used herein, the term "drug" refers to a substance used for diagnostic, cure, mitigation and/or prevention of disease. Examples of drugs are well known in the art, including but not limited to antidepressants, barbiturates, anti-hypertensives, antacids, analgesics, antibiotics, anticonvulsants, antihistamines, steroids, antipsychotics, antidiabetics, laxatives, and the like. Examples of drugs specifically administered for diseases of the liver are cyclooxygenase-2 (COX 2) inhibitors such as suldinac for prevention of hepatocarcinoma, antifibrotic drugs for treatment of cirrhosis, antioxidants for treatment of alcoholic hepatitis, and anti-viral drugs such as Lamividine (Example 1).

Preferably, contacting the hepatocyte with ligand- and substrate-coated beads of the present invention is effected in a manner suitable for inducing endocytosis of said beads, for example, in the presence of 5-Aza-2 Deoxycytidine and a solution mediator, such as DMSO, as described herein (Example 1).

According to another aspect of the present invention, there is provided a composition of matter comprising a bead coated with a hepatitis viral protein and a bioactive substance, wherein the size and/or shape of the bead are selected to be suitable for undergoing endocytosis. The hepatitis viral proteins coated onto the beads can be HBV-HBsAg or a portion thereof, and HCV E2 envelope protein or a portion thereof.

As used herein, the term "bioactive substance" refers to a substance exhibiting a biological activity. Examples of such bioactive substances that can be used in the formulation of compositions of matter of the present invention are anti-viral and anti-cancer drugs described hereinabove.

Compositions-of-matter of the present invention can be used, for example, for investigation of effect of the bioactive substance on hepatocytes in

vitro, and for therapeutic administration of the bioactive substance to hepatocytes in vivo, to subjects in need of the bioactive substance, as described in detail hereinabove.

The compositions of matter of the present invention can be administered to an individual per se or as a part of a pharmaceutical composition which includes a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of the pharmaceutical composition of the present invention is to facilitate administration of the bound bioactive substances to hepatocytes of an organism.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration of beads may, for example, include intravenous, intramuscular, sub-cutaneous, rectal, buccal and vaginal application.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of

conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

The active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. Penetrants appropriate to the barrier to be permeated may used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical composition of the present invention may be formulated in rectal and vaginal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

The pharmaceutical compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may be accompanied by instructions for administration. The pack may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting.

Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide

Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Materials and methods

Cell Culture

HepG2 and HEK 293 cells were maintained in Dulbecco's modified eagle's minimal essential medium (DMEM) (GIBCO Laboratories Grand Island New York 14072, USA) containing penicillin (100U/ml and Streptomycin 100μg/ml, supplemented with 8% fetal calf serum (GIBCO Laboratories Grand Island New York 14072, USA). Transfections of the HEK 293 cells were carried out by the CaPi method as previously described (Haviv, I et al Mol Cell Biol 1995;15:1079-1085): Cells were seeded 8 to 12 hours prior to transfection. About 60% confluent plates were transfected with the desired plasmids. Carrier DNA plasmid was added to reach the final amount of 20μg total DNA per 10 cm-diameter plate.

5-Aza-2 Deoxycytidine and DMSO Pretreatment of HepG2 cells

For 5-Aza-2 Deoxycytidine and DMSO pretreatment, HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories Grand Island New York USA) containing penicillin (100U/ml) and streptomycin (100 μg/ml) supplemented with 8% fetal calf serum (GIBCO

Laboratories Grand Island New York USA). Cells (10^6 per 10 cm dish) were seeded on glass coverslips in a 12 well plate (Corning cat #3513, Corning NY USA) in 1ml culture medium. 8 hours later 20 µl of 5mM 5-Aza-2 Deoxycytidine (AzaDC) (Sigma-Aldrich Corp., St Louis, MO, USA) in 100% DMSO (Merck) were added into each well, producing a final concentration of 100 µM 5-Aza-2 Deoxycytidine and 2% (V/V) DMSO. The culture medium was replaced every 72 hours with identical, fresh medium. The cells were maintained in medium supplemented with AzaDC and DMSO for 10-14 days before infection. Where indicated (DMSO treatment), AzaDC was omitted from the medium.

Production and purification of HBV SVPs and HCV surface proteins

For production of SVPs Chinese Hamster Ovary cells (CHO) were stably transfected with the AL26 plasmid and high HBsAg producing cells were isolated. The culture medium was collected and centrifuged (Rotor SS34 17K, 30 min. at 4°C) to remove cellular debris, and the recombinant SVPs were fractionated on CsCl gradients. HBsAg level in the resulting fractions was determined by RIA using ^{125}I labeled polyclonal αHBsAg antibody (BTG, Rehovot, Israel). Fractions enriched for HBsAgs were collected and SVPs were enriched by centrifugation through a 30% (W/V) sucrose cushion in PBS (16 hr, 27K, 4°C, SW 28 rotor). Production and purification of SVPs was performed by Biotechnology Israel (BTG, Rehovot, Israel). Sub-viral particles containing only the small HBsAg (sSVP) were produced by transfection of HEK 293 cells, as described hereinabove. 6 days post transfection the culture media was collected and centrifuged (17K, 30 min. at 4°C), to remove cellular debris. The supernatant was then layered on top of a 30% (W/V) sucrose cushion (prepared in PBS) and fractionated by 16 hours ultra centrifugation (27K, 4°C, SW 28 rotor). The pellet was resuspended in PBS, and analyzed for sSVP, by Western immunobloting using polyclonal anti SVP antibodies (BTG, Rehovot, Israel).

Recombinant preS1 protein (WT or mutant) was produced using the pRSETB preS1 vector in BL-21::pLysS bacteria as previously described (Haviv, I et al EMBO J 1996;15:3413-3420). Protein expression was induced at OD_{600} 0.8, by 1mM IPTG for 30 min. at 37°C. Cells were collected, washed and lysed by sonication in 30 ml per 500 ml original culture volume of lysis buffer (25mM Hepes-KOH pH 7.9, 5mM $MgCl_2$, 200mM NaCl, 10mM β-mercaptoethanol, 10% glycerol, 200mM PMSF, 100mM Benzamide and 10mM Benzamidine). Soluble proteins were separated from debris and inclusion bodies by centrifugation in a SS-34 rotor (16,000g 30 min.), preincubated with 10mM Imidazole and loaded on 1ml NiNTA agarose column (Qiagene Inc, CA USA) pre-equilibrated with the equivalent buffer containing 10mM Imidazole. Non-specific proteins were removed by washing with 150 column volumes of lysis buffer containing 10mM Imidazole. The preS1 proteins were step-eluted in lysis buffer containing 150mM Imidazole, and dialyzed against PBS. The identity and homogeneity of the purified proteins were determined by Coomassi staining and Western immunobloting.

Recombinant HCV E2 protein was prepared by the baculovirus system (XTL Biopharmaceuticals Ness-Ziona Israel).

Production of purified, defined TC HBV viral particles

Huh7 cells were transfected with wild type (wt) HBV DNA. At 10 days posttransfection, the culture media was collected, and viral particles were concentrated through a 30%(W/V) sucrose cushion. The pellet was re-suspended in PBS and layered on top of a preformed 20-65% (W/V) sucrose gradient (in PBS) and ultracentrifuged at 140,000g (27000 RPM) for 16 hrs at 4°C in an SW28 rotor. Fractions of 1 ml were collected from the bottom of the tube and the sucrose concentration determined by refractometer. The fractions were concentrated by ultracentrifugation through a 20% (W/V) sucrose cushion in PBS in SW28 rotor at 140000g (27000 RPM) for 16 hrs at 4°C, re-suspended in PBS and kept in 4 °C for further analysis. For infection studies, the HBV pellet was treated with DNase-I, reconcentrated by ultracentrifugation

and resuspended in PBS. To determine virus titer, viral DNA was extracted from the samples by QIAamp DNA Blood Mini Kit (QIAGEN Inc, CA USA) according to the manufacturer's protocol. The DNA was then purified on a QIAamp (QIAGEN Inc, CA USA) column and amplified by PCR. Predetermined concentrations of HBV DNA, in a cloning vector were used as a calibration standard.

Preparation of conjugated beads

Polybead amino microspheres (mean diameter 6μm) (Polysciences, Wilmington PA, USA) were conjugated with the indicated proteins according to the manufacturer protocols. Briefly, 10^8 beads were washed 3 times with PBS at pH 7.4, incubated with 8% glutaraldehyde in PBS for 16 hrs at room temperature with gentle mixing, washed with PBS and incubated with the desired protein for 5 hrs at room temperature. The beads were then incubated with 0.5M ethanolamine in PBS for additional 30 min, followed by an additional 30 min incubation with 10 mg/ml BSA in PBS, resuspended in a storage buffer containing 10 mg/ml BSA, 0.1% NaN_3 and 5% glycerol in PBS, pH 7.4 and stored at 4°C until used.

Antibodies and peptides

Polyclonal Goat antibodies against SVPs (αS) were obtained from BTG (Bio-Technology General, Israel), diluted 1:3000 in PBS for antibody-mediated blocking assays or in PBS (0.1%)- Tween 20 + 4% dried low fat milk for Western analysis. The monoclonal mouse anti preS1 antibody (MA 18/7) (IgG 0.5mg/ml) was diluted 1:5000 in PBS for antibody mediated blocking assays. The monoclonal mouse anti 6His (Sigma Israel Chemicals LTD, Rehovot, Israel) antibody was diluted 1:3000 for Western analysis. Monoclonal mouse anti HBcAg antibodies were generated by injection of bacterially expressed FPLC purified HBV core particles and selection of hybridoma secreting αHBcAg antibodies. Polyclonal rabbit antibodies against HBV core protein were generated by repeated injection of bacterially expressed, FPLC-purified

HBV core particles. For antibody mediated blocking assays the ascites fluid was diluted 1:5000 in PBS. For Western analysis horseradish peroxidase-conjugated donkey anti goat and goat anti mouse antibodies (Jackson Immunoresearch Laboratories, USA) were used. Cadherin was detected with Anti Pan-Caderin antibody (Sigma-Aldrich Corp, St Louis MO, USA).

A synthetic peptide comprising the 21-47 amino acids of HBV preS1 PLGFFPDHQLDPAFGANSNNPDWDFNPGK (NCBI Accession No. AB056513.1, SEQ ID NO: 1) was synthesized (Weizmann Institute of Science Internal Services, Rehovot, Israel), purified by HPLC using a C18 column (Amersham Pharmacia Biotech UK LTD), lyophilized and resuspended in PBS before use. The peptide composition was confirmed by amino acid analysis (Weizmann Institute of Science internal Services, Rehovot, Israel).

Bead- Mediated Attachment (BMA) and competition assays

HepG2 cells were seeded as described hereinabove, $2X10^6$ beads were added to the cells and incubated for additional 14 hours. For competition assays the appropriate competitor antigens were added two hours prior to the addition of the conjugated beads. The unbound beads were removed and cells were fixed in 4% paraformaldehyde solution and mounted in Aqua-polymount (Polysciences, Wilmington, PA, USA). Attached beads were visualized, documented and quantified by DIC light microscopy (Zeiss Axiophot). The average number of beads per cell was evaluated by monitoring 300 cells from 7-10 representative fields. Attachment efficiency was quantified for each representative microscopic field and the average attachment efficiency, expressed as the number of beads per cell, was determined. The results were grouped into the following categories: cells to which no beads attached (0 beads per cell), cells that attached 1-4 beads per cell and cells that attached more then 4 beads per cell (> 4 beads per cell). Average attachment efficiencies for each group are presented together with the standard deviation.

HBV infection and DNA analysis

HBV-positive sera were used to infect cells as previously described (Gripon, P et al J Virol 1988;62:4136-4143; Pugh, J.C. and Summers, J.W. Virology 1989;172: 564-572; and Tuttleman, J.S. et al J Virol 1986; 58: 17-25). Briefly HepG2 cells were cultured on glass coverslips (18 mm in diameter, No.1) in a 12 well plate (AS ABOVE), with or without additional 2% DMSO for 6-10 days. Cells were incubated with HBV positive sera (Israel Blood Bank) (10^9 particles/ml) for 14 hours at 37°C, extensively washed with PBS and fresh medium added with or without DMSO or 5-Aza-2 Deoxycytidine. For PCR analysis, cells were washed extensively 4 days post-infection in PBS, collected by rubber-policeman, and lysed in a Proteinase K lysis buffer for 6 hrs in 37°C, extracted with phenol-chloroform and ethanol precipitated. Detection of cccDNA and RC-DNA was performed as described (Ilan, E et al Hepatology 1999;29:553-562). DNA was quantitated by semi quantitative PCR analysis of a cellular gene (AML-2) ensuring that equal amounts of DNA were used for HBV RC- and cccDNA analysis. Similar analysis was performed with the secreted virions.

For analysis of HBV replicative DNA forms, infected cells were harvested at the indicated time intervals and total and extrachromosomal DNA extracted and analyzed by agarose gel electrophoresis and Southern blotting (Tuttleman, J.S. et al J Virol 1986; 58: 17-25). HBV sequences were detected using ^{32}P labeled DNA probes synthesized by random priming from an intact HBV DNA genome template.

For detection of viral proteins, cells were washed 4 times in cold PBS four days post-infection, fixed on slides as described hereinabove, and incubated with polyclonal αS (HBsAg) and αCore (HBcAg) antibodies prepared as described hereinabove. Immunodetection was performed using Fluorescein (FITC) conjugated anti Goat and Rhodamine-Red-X (RRX) conjugated anti rabbit second antibodies (Jackson Immunoresearch Laboratories, USA). Slides were mounted in Aqua-polymount (Polysciences,

Wilmington PA, USA). Microscopic images were obtained using a Bio-Rad MRC-1024 confocal system, utilizing an Argon/Krypton mixed gas laser and mounted on a Zeiss Axiovert microscope.

Scanning electron microscopy (SEM)

Cells were cultured on glass coverslips (13 mm in diameter, No.1, Marienfeld, USA) in a 12 well plate, for at least 4 days with or without DMSO, and incubated with protein-coated beads for 16 hrs. Cells were then washed extensively with DMEM prewarmed to 37°C, fixed for 1 hr with Karnovsky's fixative (3% paraformaldehyde, 2% glutaraldehyde, 5mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4 containing 0.1 M sucrose). Cells were rinsed and post-fixed for 1 hr with 1% osmium tetroxide in 0.1 M cacodylate buffer, incubated with 1% tannic acid in water and then with 1% uranyl acetate in water. All buffers and DMEM were freshly prepared and filtered (0.2μm filter) before use. The slides were rinsed, dehydrated with a graded ethanol series, critical-point dried (Pelco CPD2 International, Redding CA USA) and sputter coated with gold for SEM analysis (S150 Edwards, USA). The specimens were examined at an accelerating voltage of 20-25 kV using a JEOL GMC 6400 scanning electron microscope.

Transmission electron microscopy

For transmission electron microscopy, cells were seeded in 35 mm Falcon dishes (Becton, Dickinson and Co, Discovery Labware, Bedford, MA, USA) and incubated for 4 days with or without DMSO, before adding SVP protein-conjugated beads for an additional 16 hrs. Cells were fixed in Karnovsky's fixative, post fixed with 1% osmium tetroxide, 0.5% potassium dichromate and 0.5% potassium hexacyanoferrate in 0.1M cacodylate buffer. The fixed cells were then stained *en bloc* with 2% aqueous uranyl acetate, dehydrated with ethanol dehydration and the dishes embedded in Epon 812 (Epon, Tuosimis, MD). Sections were prepared using a diamond knife (Diatome, Biel) and examined using a Philips CM-12 transmission electron

microscope at an accelerating voltage of 100KV. SVP were visualized by negative staining (Weizmann Institute Internal Services, Rehovot Israel).

Example 1

5-Aza-2 Deoxycytidine and DMSO facilitate HBV infection of HepG2 cells

Efficient HBV infection of cultured human hepatic cells is a primary requirement for effective investigation of virus-cell interactions, and prevention and treatment of the disease. However, established human hepatocyte lines such as HepG2 and Huh-7 are refractive to HBV infection. In primary liver culture cells, but not established lines, treatment with 1.5-2% DMSO results in enhanced and prolonged HBV (Gripon, P et al J Virol 1988;62:4136-4143) and DHBV infection (Pugh, J.C. and Summers, J.W. Virology 1989;172:564-572).

To investigate whether treatment with DMSO can enhance susceptibility and efficiency of HBV infection in established cell lines, DMSO-treated (2%) and untreated HepG2 cells were incubated with HBV positive human serum as described hereinabove. Four days later cells were analyzed by indirect immunofluorescent staining for the presence of viral proteins. Surprisingly, viral-specific antigens HBcAg and HBsAg were both detected in the DMSO-treated cells, whereas untreated cells were barely reactive (Figure 1A). Thus, treatment with 2% DMSO enables efficient HBV infection of the HepG2 cells.

In order to determine whether the DMSO-facilitated infection in HepG2 cells also gives rise to viral replication, total DNA was extracted from the infected cells and from the HBV positive sera virus stock. Equal amounts of DNA were analyzed by PCR for the presence of HBV relaxed-circular (RC) and cccDNA, a marker of productive infection and viral genome replication. RC-DNA was detected both in the infected cells and in the virions whereas cccDNA was detected only in the cells (Figure 1B, lane 2). No viral DNA of any form was detected in the DMSO-untreated cells, despite careful maintenance of equal amounts of DNA (see AML-2, Figure 1B, lower panel).

To directly assess the presence of HBV replication forms, untreated and DMSO-treated cells were harvested 5, 9 and 14 days after infection, and DNA from the cells was analyzed for RC, single-stranded (SS) and cccDNA, by agarose gel electrophoresis and Southern blot hybridization. Remarkably, already 5 days after infection significant amounts of the DNA replication markers were detected only in DMSO treated cells (Figure 1C, lane 10). HBV DNA was even more abundant at 9 and 14 days post infection (Fig 1C, lanes 5-7).

Significantly, the replicative intermediate DNA forms could not be detected in cells treated with Lamivudine (3TC), a potent HBV RTase inhibitor (Fig 1C, lane 8), indicating that viral DNA replication is the result of reverse-transcriptase activity. Furthermore, a neutralizing antibody raised against the 21-47 amino acids region of preS1 (MA 18/7) (Neurath, A.R. et al Vaccine 1989;7: 234-236) effectively blocked infection in the DMSO-treated HepG2 cells as well (Fig 1C lane 9). Thus, Hep G2 cells pretreated by DMSO efficiently support both HBV infection and replication, mimicking natural infection.

Surprisingly, it was uncovered that pretreatment of the cultured hepatocytes with the methylation inhibitor 5-Aza-2 Deoxycytidine and DMSO results in even greater efficiency of infection of HepG2 cells by HBV. Hep G2 cells pretreated extensively with 100 mM 5'-Aza-2'-Deoxycytidine and 2% DMSO exhibited 100% infection by both serum derived HBV (Figure 1D, Serum HBV, right column) and pure virus particles (Figure 1D, TC HBV, left column), expressing both the HBcAg and HBsAg viral proteins (Figure 1D, Merge).

Example 2

DMSO-treated HepG2 cells bind recombinant viral protein-conjugated beads

Preparation of HBV sub-viral particles (SVP) for Bead-Mediated Attachment (BMA) Assay

Due to the paucity of pertinent experimental data, the underlying mechanisms of HBV infection of cultured hepatocytes cell lines have remained unclear. In order to elucidate the nature and action of the involved receptors [specifically, whether the DMSO-mediated enhancement of infection requires induced expression and presentation of differentiation-specific viral receptors (receptor activation)] and provide a quantitative measure of virus attachment, the bead-mediated attachment (BMA) assay that measures viral attachment at single cell resolution was developed.

Recombinant HBV sub-viral particles (SVP) were produced in animal cells by utilizing the AL26 plasmid that contains an integrated form of HBV DNA (Faktor, O et al Virology 1988;162:362-368). Chinese Hamster Ovary (CHO) cells were transfected with AL26 plasmid and high HBsAg producer lines were established that secrete HBsAg sub-viral particles (SVPs) with the expected 1.22 gr/ml density (Figure 2A). Electron microscopy revealed that the particles are spherical and homogeneous in size (22nm in diameter). For protein composition analysis, ^{35}S metabolic labeled particles were prepared and analyzed by SDS-PAGE. The small HBsAg p24 and p28 are the major components, the middle preS2 proteins, p33 and p36, the minor components (Figure 2B and 2C), and the large preS1 proteins are barely detectable. However the fact that these particles contain all the three HBV protein components was confirmed by immunoblotting utilizing different specific antibodies (Figure 2B, right panels). Taken together, these results clearly show that the structure, density and composition of the purified recombinant SVP are similar to that reported for serum derived 22nm SVPs.

DMSO improves SVP-beads attachment

Conjugation of ligands to synthetic beads has been employed to study adhesion-mediated interactions (see, for example, Levenberg S et al J Cell Sci 1998;111: 347-57). Neurath et al has used synthetic (U.S. Pat. Nos. 5,620,844 and 5,565,548 to Neurath et al) and magnetic beads (U.S. Pat. Nos. 5,798,206 to Neurath et al) coated with HBV viral envelope proteins for detection of anti-HBV antibodies in solution. In order to determine whether HBV viral antigen-coated beads can mimic mechanisms of viral-hepatic cell interactions, recombinant SVP containing all three HBsAg components were conjugated to synthetic beads, as described in the Methods section hereinabove, to obtain SVP-beads. Control BSA-conjugated beads were also prepared and both were incubated for 16 hrs with untreated HepG2 cells. The unbound beads were removed, the cells fixed and the number of the attached beads per cell was determined by light microscopy. The percentage of the cells that bind SVP-beads (50%) was significantly higher than the control BSA-beads (18.7%) (Figure 3A, 3B). Remarkably, pretreatment with DMSO significantly improved SVP-beads attachment, with nearly all (93%) of the treated cells binding beads, as compared to 50% of untreated cells (Figures 3C and 3D). Moreover, about 49% of the treated cells bind more than 4 SVP-beads per cell (Figure 3D), 244 fold higher than the binding in untreated cells (0.2%, Figure 3B). Furthermore, the DMSO-mediated enhancement in SVP-beads attachment is specific, as the majority (56%) of the cells bind no BSA-beads at all and only 0.8% bind more than 4 beads per cell. Significantly, the SVP-beads attach poorly to non-liver cells such as Cos-1 and HeLa cells (data not shown), suggesting that SVP-beads attachment, like viral attachment, is highly cell type specific. Thus, these results demonstrate, for the first time, that pretreatment of cultured hepatocytes with DMSO not only increases the number of cells that bind HBsAg sub-viral particles but also enhances the binding capacity of a single cell. Similar enhancement of binding to Hep G2 cells was observed with recombinant HCV E2 antigen conjugated to the synthetic beads (Figures 9A and 9B). Thus,

treatment of cultured cells with DMSO strongly enhances both HBV infection, and SVP-bead, and HCV E2 attachment, indicating activation of hepatotrophic virus receptors on the cell membrane.

Example 3

Binding and endocytosis of recombinant viral protein-conjugated beads by DMSO-treated HepG2 cells

Endocytosis of SVP-coated beads

In order to determine whether SVP-coated beads can efficiently mimic internalization of viral particles into the hepatic cell, the effect of bead attachment on the cell surface was further examined by transmission electron microscopy (TEM, Figures 4A-4D). Cells binding SVP-beads show extensive membrane protrusions around the attached beads that upon long incubation are efficiently internalized into DMSO treated HepG2 cells (Figure 4C). This was hardly observed using DMSO untreated HepG2 cells or BSA-conjugated beads (not shown). Scanning electron microscopy revealed a tight association of the beads with the cell surface (Figures 4E-4H). In agreement with the TEM results, SVP-conjugated beads were partially (Figure 4G) or fully (Figure 4H) engulfed by the cells. The bead internalization, which is very likely the outcome of endocytosis, is efficient, up to eight beads being internalized within a given cell (Figure 4H).

When subjected to the same conditions, concanavalin-A conjugated beads were found to attach to the HepG2 cells efficiently, but were not internalized (data not shown). Thus, the internalization of viral protein-coated beads is a specific, recognition-dependent phenomenon. Taken together these results demonstrate that SVP–beads mimic viral-hepatic cell interaction, and are internalized after binding to DMSO-treated HepG2 cells.

Recombinant preS1 protein and cell attachment

The DMSO-enhanced binding and internalization of SVP-conjugated beads by cultured hepatic cells can be used to investigate the role of specific

viral antigens and peptide sequences in HBV infection. To locate the regions within HBsAg which play a role in cell attachment, a recombinant preS1 protein was prepared and conjugated to beads (preS1-beads). This region of the large HBsAg protein was previously shown to play an important role in receptor recognition (De Meyer, S et al J Viral Hepat 1997;4:145-153; Le Seyec, J et al J Virol 1999;73:2052-2057; and Neurath, A.R. et al Cell 1986;46:429-436). Untreated cells (Figure 5B) did not bind preS1-beads efficiently. However, DMSO treatment improved binding by greater than 50 fold (Figure 5A, SVP). Thus, pretreatment of Hep G2 cells with DMSO enhanced the specific binding of the preS1 region of HBsAg, as demonstrated by the bead-mediated assay (BMA). Interestingly, although the number of recombinant preS1 molecules per bead far exceeds that of SVP per bead (data not shown), the maximal attachment efficiency of preS1-bead binding is about two folds lower than that of SVP-beads.

The 21-47 amino acids region of preS1 can block HBV attachment to HepG2 cells. Antibodies raised against this epitope have neutralizing activity (Figure 1C; and Neurath, A.R. et al Vaccine 1989;7: 234-236). In order to determine whether the BMA assay can be employed to screen potential HBV therapeutic and diagnostic agents, a corresponding synthetic peptide was prepared and analyzed using BMA analysis. Here again DMSO dramatically improved the attachment of these beads but only about 18% of the cells bound 4 or more beads (Figure 5A), comparable to the results of recombinant preS1-beads binding (Figure 5A). Taken together, these data indicate that the attachment activity of the preS1 region is most likely found within the 21-47 amino-acids sequence, that SVPs are twice as efficient as preS1 protein in cell attachment and that the BMA mimics natural HBV infection processes.

As described hereinabove, HepG2 cells can be made even more susceptible to HBV infection by addition of 5'-Aza-2'-Deoxycytidine to DMSO. Similarly, treatment of the cells with 2% DMSO+100 mM 5'-Aza-2'-

Deoxycytidine resulted in a two-fold increase in HBV preS1-attachment to HepG2 cells over that attained with DMSO treatment alone.

Fine mapping of the preS1 major attachment-determining region with BMA

The major antigenic determinant of the HBV infection neutralizing MA 18/7 monoclonal antibody is the preS1 DPAF sequence (Figure 2C), suggesting that this sequence may be important for attachment. In order to directly investigate the role of preS1 sequences in HBV infection, a recombinant preS1 protein with a scrambled QLDPAF epitope was prepared and tested for attachment in the BMA (Figure 6A). The wild type and scrambled mutant proteins are His-tagged preS1 proteins and are both reactive to the His-tag specific antibody α 6 His (Figure 6B). However the anti-preS1 MA 18/7 antibody did not recognize the mutant protein. The wild type and scrambled mutant preS1 proteins were conjugated to beads and subjected to BMA assays. In contrast to wild type, the scrambled mutant is inefficient in attachment (Figure 6C). Furthermore, a soluble fraction of the scrambled mutant (mut) cannot block the binding of wild type (WT) preS1-beads to cells (Figure 6C). Thus, BMA analysis clearly identifies the preS1 QLDPAF motif as the dominant HBV attachment site.

Interestingly, sequence comparison with other HBV proteins revealed that the X protein of HBV contains a sequence homologous to the major preS1 attachment epitope (QLDPS/AR) (Figure 6D). Surprisingly, it was found that HBV pX can also be immunoprecipitated by the MA 18/7 anti preS1 antibody (unpublished data), indicating that pX might also be involved in cell attachment. Furthermore, of the two HepG2 EGF-repeat proteins that bind recombinant preS1, and are induced by DMSO, one has been reported to bind pX (Sun BS et al, Hepatology 1998;27:228-39). A search of the protein sequence database revealed that this minimal epitope is shared by other viral, bacterial and cellular proteins that participate in cell adhesion, attachment and fusion (Figure 6D). Thus, the BMA assay successfully identified the QLDPAF

sequence, or a portion thereof, as a general motif critical to HBV adherence and attachment to hepatic cells in HBV infection.

Example 4

BMA analysis of small HBsAg reveals a secondary non-preS1 attachment region and multivalent interaction in HepG2 binding

Cooperation in attachment between preS1 and non-preS1 components of the small HBsAg has been reported (see, for example Breiner, K.M. et al J Virol, 1998 72, 8098-8104, Gripon, PJ et al Virol 1988;62:4136-43, Neurath A.R. et al Cell, 1986 46, 429-436 and Urban, S et al EMBO J,2000;19:1217-1227). As demonstrated hereinabove, both recombinant preS1 and synthetic 21-47 amino-acids peptide show only 50% attachment activity of the SVP-beads, strongly indicating the presence of a second region that plays a role in this process. To determine the location of the second SVP attachment site, HBV sub-viral particles were prepared, composed of the small HBsAg and lacking the preS1 and preS2 regions (Figure 7A, sSVP,). Surprisingly, the non-preS1 and non preS2 beads display specific attachment in a DMSO-dependent manner (Figure 7B). However, binding efficiency of the sSVP-beads (sSVP +, Figure 7B) was about 5 times lower (7.9% vs. 48.8%) than that obtained with SVP beads bearing the preS1 domain (SVP+, Figure 7B). Thus, using the BMA assay it was revealed that small HBsAg has an additional and independent attachment site that interacts with a distinct cellular receptor.

Synergistic cooperation between multivalent SVP attachment sites

As detailed hereinabove, attachment efficiency of the preS1 region is only 50% that of SVP-beads (Figure 5A). Yet, particles lacking the preS1 and preS2 regions exhibit only 8% attachment activity (Figure 7B). This implies that either additional, undetected attachment epitopes are present or that the two regions identified by BMA analysis act in a synergistic manner. Using the BMA assay and ligand competition with soluble recombinant preS1 as a competitor, the contribution of the preS1 domain to SVP binding was

investigated. HepG2 cells were preincubated with increasing amounts (0- 500 μg/ml) of the preS1 protein prior to the addition of the conjugated beads (Figure 8A, lower panel). The preS1-beads attachment was completely blocked by the homologous soluble preS1 protein (Figure 8A, lower panel). Interestingly, SVP-beads attachment was also competed by preS1 protein, but about 8% of the cells continued to show efficient SVP attachment even in the presence of vast excess of the competitor (Figure 8A lower panel). Under similar conditions the attachment activity of the small SVP beads (sSVP) remained refractory to competition by the preS1 protein (Figure 8A, right upper panel), suggesting that this region binds a distinct receptor on the cell surface.

In order to further characterize this non-preS1 and non-preS2 binding, the BMA assay was combined with attachment-neutralizing experiments using the anti preS1 M/A 18/7 neutralizing monoclonal antibody. As is demonstrated in Figure 8B, 2 hours incubation of the protein-conjugated beads with the monoclonal MA 18/7 antibody prior the BMA assay effectively blocks 100% of preS1-bead attachment (Figure 8B, left panel), but only about 80% of SVP-bead attachment (Figure 8B, right panel). Significantly, here again about 8% of SVP-bead attachment to the cells could not be blocked (Figure 8B, right panel). Control anti HBcAg monoclonal antibody showed no significant inhibition of SVP-bead binding.

Both the competition assays and the combined BMA- neutralizing antibody assay demonstrate that the preS1 epitope provides the major attachment epitope. As the small SVP-beads consistently demonstrate about 8% attachment activity (Figure 7B), the same level of activity that remains refractory to competition, it is clear that other HBsAg regions, like the preS2, play a minor role. Collectively, these results strongly indicate that distinct attachment regions of HBV surface antigen particles are likely to act in a synergistic manner, whereby numerous non-preS1interactions with the cell membrane act to facilitate strong preS1 binding.

Thus, treatment of cultured hepatic cells with DMSO renders them susceptible to active infection with HBV, and binding-internalization of recombinant viral protein-conjugated beads, providing, for the first time, a hepatic cell culture system for in vitro infection with HBV and other viruses, an efficient attachment assay for diagnostic, clinical and research applications, and novel methods for delivery of various substances to cells. Furthermore, using the methods of the present invention, the importance of the QLPD epitope to cell-virus, virus-virus and cell-cell interactions was uncovered.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A method of obtaining cultured hepatocytes susceptible to viral infection, the method comprising exposing the cultured hepatocytes to 5-Aza-2 Deoxycytidine and a solution mediator, to thereby obtain cultured hepatocytes susceptible to viral infection.

2. The method of claim 1, wherein said solution mediator is DMSO.

3. The method of claim 1, wherein said hepatocytes are HepG2 cells.

4. The method of claim 1, wherein said hepatocytes are human hepatocytes.

5. The method of claim 1, wherein said viral infection is an HBV infection.

6. The method of claim 1, wherein said viral infection is an HCV infection.

7. A method of quantifying binding between a ligand and a hepatocyte, the method comprising:

(a) contacting the hepatocyte with beads coated with the ligand; and

(b) determining the amount of said beads bound to, or present in the hepatocyte to thereby quantify binding between the ligand and the hepatocyte.

8. The method of claim 7, wherein the hepatocyte is a cultured hepatocyte sensitized by contacting the hepatocyte with 5-Aza-2 Deoxycytidine and a solution mediator, to thereby obtain a sensitized hepatocyte.

9. The method of claim 8, wherein said solution mediator is DMSO.

10. The method of claim 7, wherein the hepatocyte is a cultured hepatocyte.

11. The method of claim 7, wherein the hepatocyte is a human hepatocyte.

12. The method of claim 7, wherein the ligand is a viral protein.

13. The method of claim 12, wherein said viral protein is HBsAg or a portion thereof.

14. The method of claim 7, wherein the ligand is a synthetic peptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

15. The method of claim 7, wherein said beads are selected from the group consisting of polystyrene beads, dextran beads, glass beads and metal beads.

16. The method of claim 7, wherein quantifying said beads is performed using a method of detection selected from the group consisting of microscopy, radioactivity, fluorescence, magnetic polarity, color detection and immuno-affinity.

17. A method of delivering a substance into a hepatocyte, the method comprising contacting the hepatocyte with a bead coated with:

(a) a ligand capable of binding the hepatocyte; and

(b) the substance;

thereby introducing the substance into the hepatocyte.

18. The method of claim 17, wherein said contacting the hepatocyte is performed in vivo.

19. The method of claim 17, wherein said contacting the hepatocyte is performed in vitro.

20. The method of claim 19, wherein the hepatocyte is a cultured hepatocyte pretreated with 5-Aza-2 Deoxycytidine and a solution mediator, to thereby obtain a sensitized hepatocyte.

21. The method of claim 20, wherein said solution mediator is DMSO.

22. The method of claim 20, wherein said cultured hepatocyte is a HepG2 cell.

23. The method of claim 17, wherein the hepatocyte is a human hepatocyte.

24. The method of claim 17, wherein said ligand is a viral protein.

25. The method of claim 24, wherein said viral protein is HBsAg or a portion thereof.

26. The method of claim 17, wherein said ligand is a synthetic peptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

27. The method of claim 17, wherein the bead is selected from the group consisting of polystyrene beads, dextran beads, glass beads and metal beads.

28. The method of claim 17, wherein the substance is selected from the group consisting of antibodies, toxins, nucleic acids and drugs.

29. The method of claim 17, wherein contacting the hepatocyte is effected in a manner suitable for inducing endocytosis of said bead.

30. A method of identifying a substance capable of modifying viral infection in hepatocytes, the method comprising:

(a) contacting the hepatocytes with beads coated with a viral ligand capable of binding the hepatocytes, wherein said contacting is performed in a presence of the substance; and

(b) determining the amount of said beads bound to, or present in the hepatocytes to thereby determine the ability of the substance to modify viral infection of the hepatocytes.

31. The method of claim 30, wherein the hepatocytes are sensitized hepatocytes obtained by contacting hepatocytes with 5-Aza-2 Deoxycytidine and a solution mediator.

32. The method of claim 31, wherein said solution mediator is DMSO.

33. The method of claim 30, wherein the hepatocytes are cultured hepatocytes.

34. The method of claim 30, wherein the hepatocytes are human hepatocytes.

35. The method of claim 30, wherein said viral ligand is HBsAg or a portion thereof.

36. The method of claim 30, wherein said viral ligand is a synthetic peptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

37. The method of claim 30, wherein said beads are selected from the group consisting of polystyrene beads, dextran beads, glass beads and metal beads.

38. The method of claim 30, wherein said determining said amount of said beads bound to, or present in the hepatocytes is performed using a method of detection selected from the group consisting of microscopy, radioactivity, fluorescence detection, magnetic polarity, color detection and immuno-affinity.

39. The method of claim 30, wherein contacting said beads is performed in vitro.

40. The method of claim 30, wherein contacting said beads is performed in vivo.

41. A composition-of-matter comprising a bead coated with:

(a) a hepatitis viral protein selected from the group consisting of HBV-HBsAg or a portion thereof and HCV E2 or a portion thereof; and

(b) a bioactive substance;

wherein a size and/or shape of said bead is selected suitable for undergoing endocytosis.

42. The composition-of-matter of claim 41, wherein said viral protein is a synthetic peptide.

43. The composition-of-matter of claim 42, wherein said viral protein is a synthetic peptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

44. The composition-of-matter of claim 41, wherein said bead is selected from the group consisting of polystyrene beads, dextran beads, glass beads and metal beads.

45. A pharmaceutical composition comprising the composition-of-matter of claim 41 and a pharmaceutically acceptable carrier.

46. The pharmaceutical composition of claim 45, wherein said viral protein is a synthetic peptide.

47. The pharmaceutical composition of claim 45, wherein said viral protein is a synthetic peptide comprising the amino acid sequence set forth in SEQ. ID. NO: 1.

48. The pharmaceutical composition of claim 45, wherein said bead is selected from the group consisting of polystyrene beads, dextran beads, glass beads and metal beads.

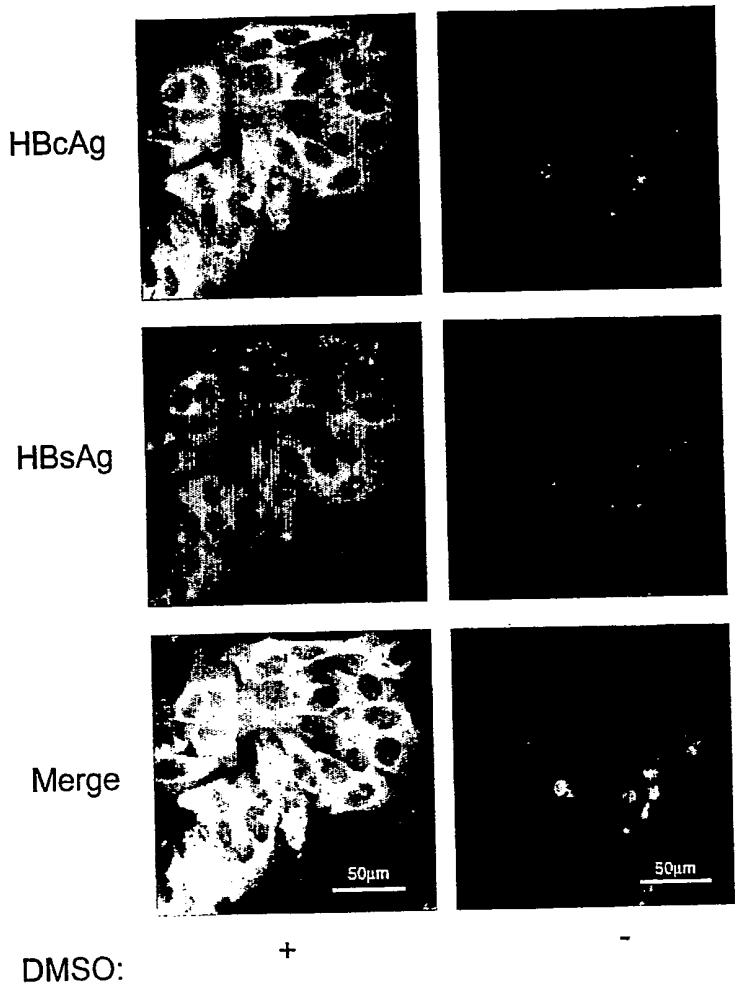

HBcAg
HBsAg
Merge
50µm
50µm
DMSO:
+
-

Fig. 1a

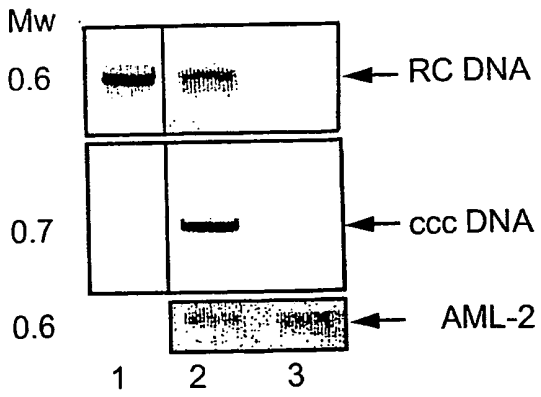

Mw
0.6
RC DNA
0.7
ccc DNA
0.6
AML-2
1
2
3

Fig. 1b

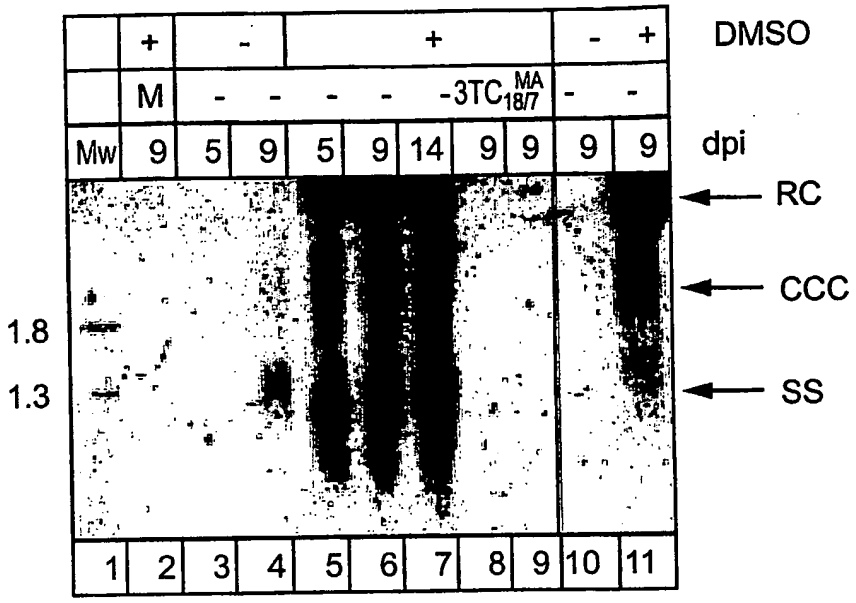

+
-
+
- +
DMSO
M
- - - - -3TC MA 18/7
- -
Mw
9
5
9
5
9
14
9
9
9
9
dpi
RC
CCC
SS
1.8
1.3
1
2
3
4
5
6
7
8
9
10
11

Fig. 1c

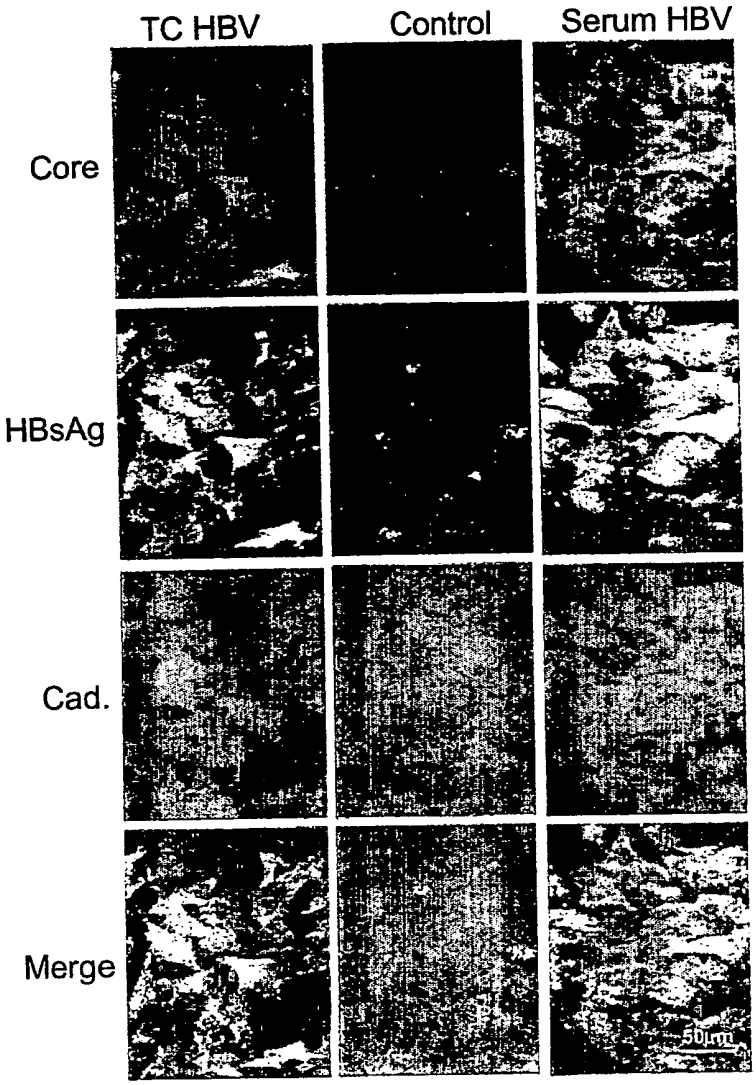


Fig. 1d

Fig. 2a

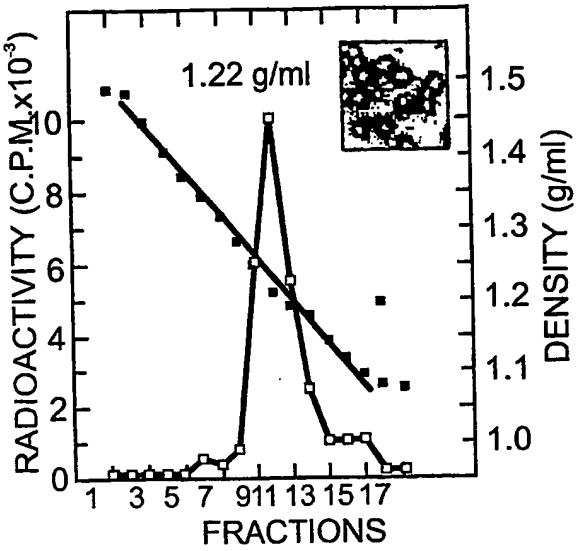


Fig. 2b

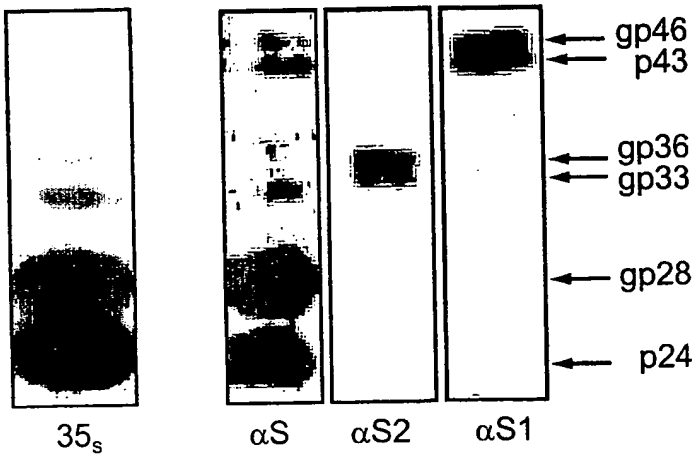
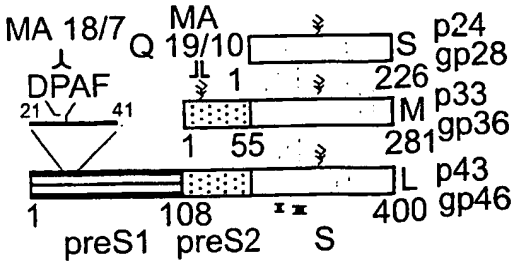


Fig. 2c



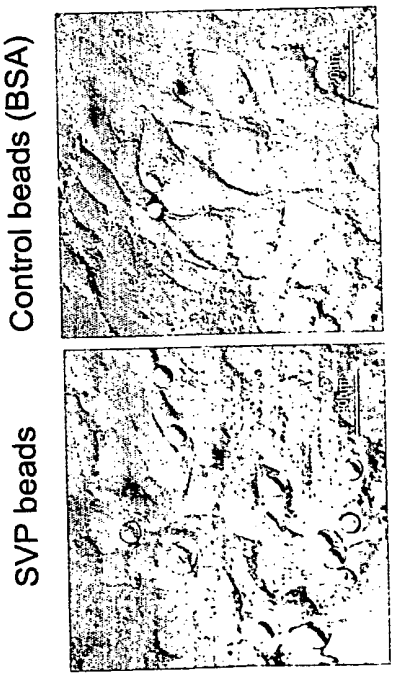


Fig. 3a

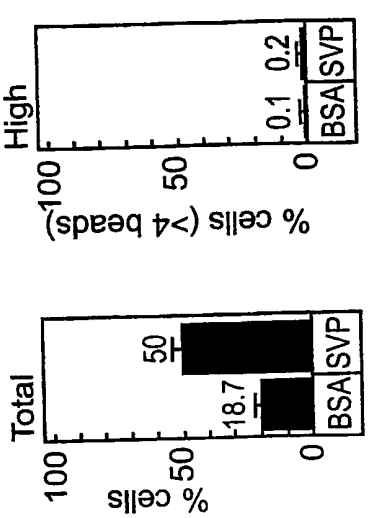


Fig. 3b

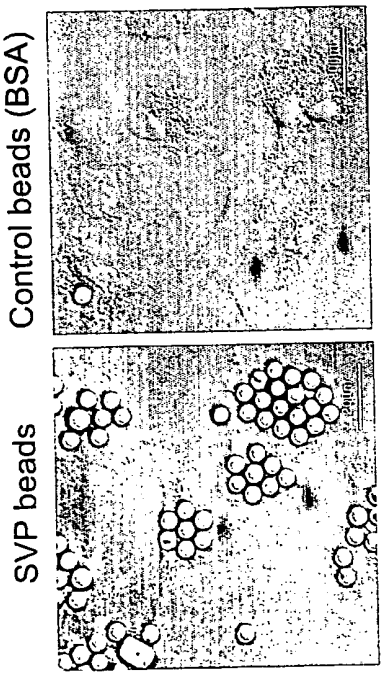


Fig. 3c

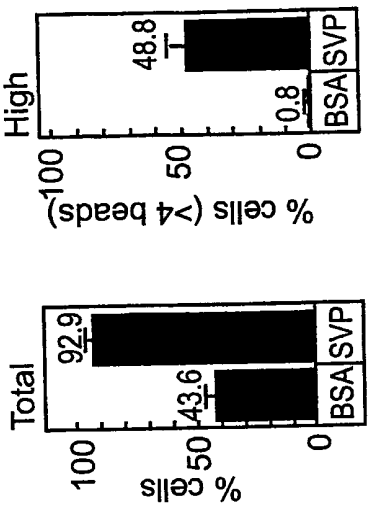


Fig. 3d

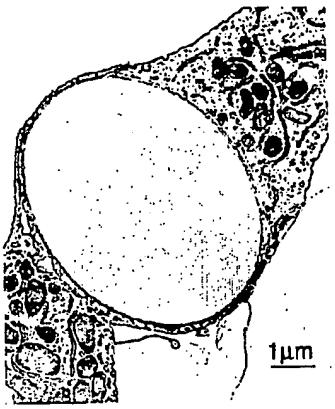


Fig. 4a

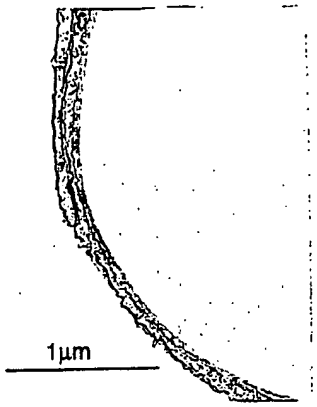


Fig. 4b

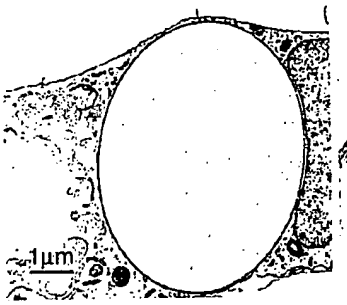


Fig. 4c

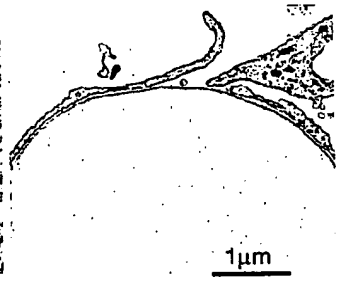


Fig. 4d

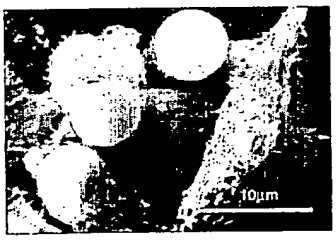


Fig. 4e

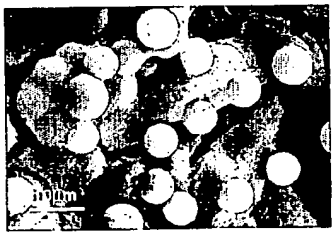


Fig. 4f

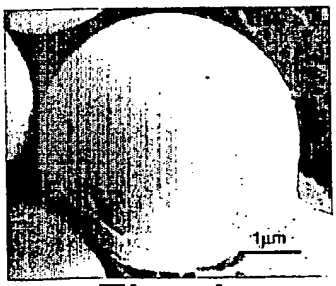


Fig. 4g

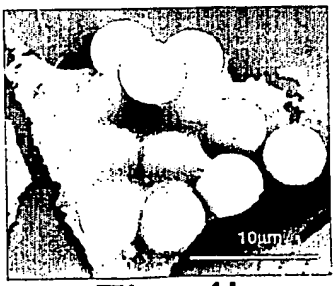


Fig. 4h

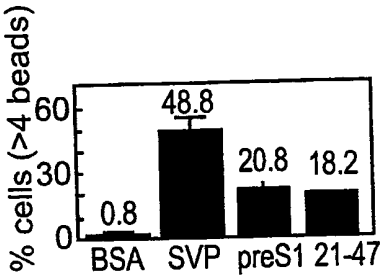


Fig. 5a

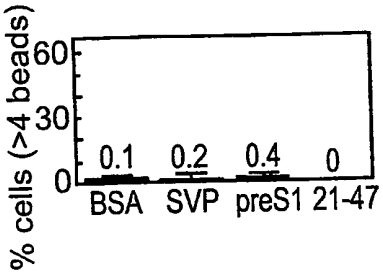


Fig. 5b

wild-type preS1 PDHQLDPAFG

mutant preS1 PDH**A**L**Q**P**D**FG

Fig. 6a

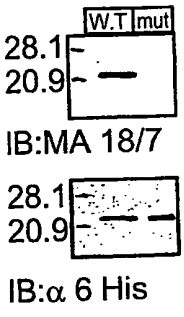


Fig. 6b

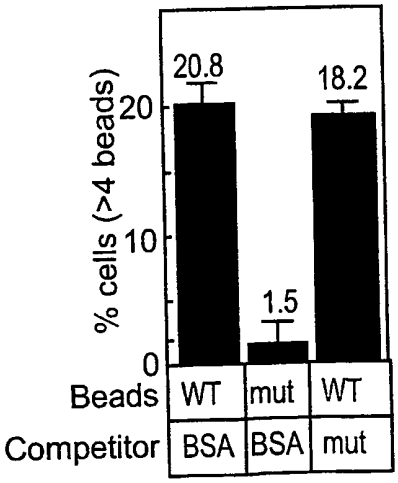


Fig. 6c

Name	Sequence	Role
A. Viral and bacterial proteins		
HBV preS1	FPDHQLDPAFGANSNN	Surface proteins, receptor binding
HBV pX	RLYCQLDPARDVLCLR	Regulatory protein
THOGV gp75	ACLRQLDPAKHWAPRK	virus endocytosis
LassaV. NC	DAMLQLDFNAKTWMDI	nucleocapsid protein
HIV vif	RYSTQLDPDLADQLIH	virus associated, infection
CMV1 L	CLRHQLDPPLLRHLDK	cell fusion and infection
CDV F2	KQHTKLDPAFKPDLTG	fusion of membranes
HPV16L2	QQVKVLDPAFVTTPTK	viral capsid protein
HSV VP22A	RAPRPTEPAFPAATGS	viral tegument
CBF1	SQCEPLDPAFSQADNE	mammalian cell fusion
B. Cellular adhesion molecules		
hPallidin	EGELQLDPAVPELFAA	fusion (TSNARE interactions)
Integrin α3	RRRRQLDPGGGQGPPP	cell adhesion to bas.Mem & matrix
Fibrillin1	CTICQLDPICGKGFSR	component of connective tissue
Bystin	FPMPQLDPRVLEVYRG	cell adhesion
Laminin B1	SLIRSSDPAFRILEDG	cell adhesion
Desmocollin	IVTKKDDPAFAKYVDD	cell adhesion
Heparin cfII	QIADFSDPAFISKTNN	heparin co-factor, rich in liver
Collagen 4α4	QKGPPGDPAFGHLGPP	basement membrane prot. adhesion
CSKP/LIN2	RRMLMLDPAERITVYE	membranal, binds syndecan, actin
AOC3/VAP1	VNHKALDPARWTIQKV	cell adhesion, also in liver
αActinin1	DRDLLLDPAWEKQQRK	cell adhesion
Cadherin 14	LAEADLDPSVPPYDSL	cell adhesion

Fig. 6d

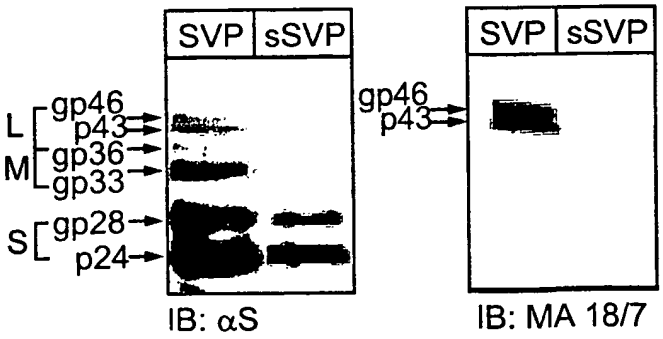


Fig. 7a

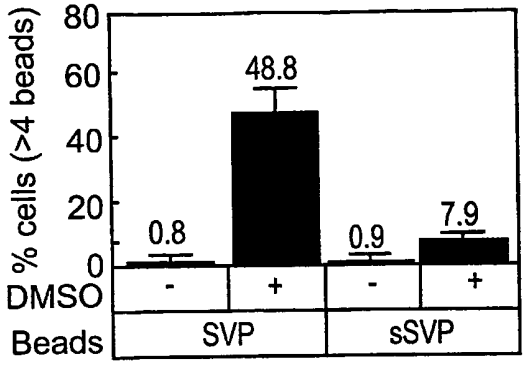
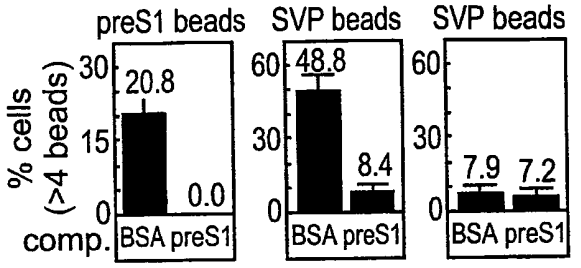


Fig. 7b



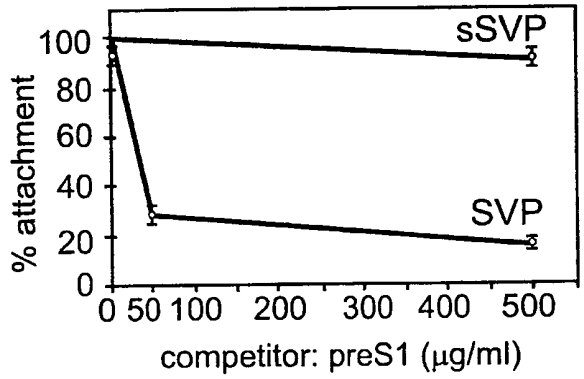


Fig. 8a

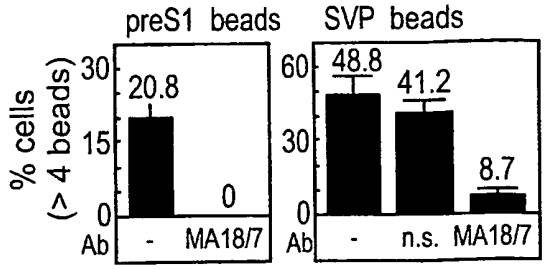


Fig. 8b

Fig. 9a

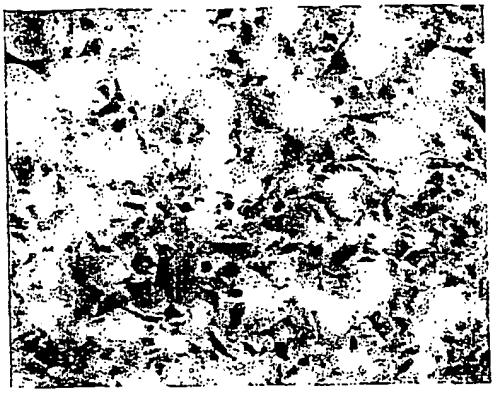


Fig. 9b

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